MODULATION OF CHOLINERGIC SYNAPTIC TRANSMISSION IN THE RAT HIPPOCAMPUS

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II. DECLARATION

In accordance with Regulation 3.8.7 of the University of Edinburgh, I declare that this thesis has been composed by myself and all the work is my own except certain results in chapter 6 which were obtained in conjunction with Diederik O. Bulters B.Sc.

Some of this work has been published as detailed in the appendix (Section 9.1).

Signature:

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31 August 1998

III. ABSTRACT

Intracellular recordings were made from pyramidal neurones in the CA1 region of the rat hippocampus to investigate aspects of cholinergic synaptic transmission in the mammalian brain. In the presence of ionotropic glutamate and γ-aminobutyric acid (GABA) receptor antagonists, single shock stimulation of the septohippocampal cholinergic input, in *stratum oriens*, evoked a slow excitatory postsynaptic potential (EPSP) which was associated with an increase in input resistance. Stimulation at intensities sub-threshold for evoking a slow EPSP caused a reduction of spike frequency adaptation (SFA). Pharmacological analysis of these responses revealed that they were mediated by muscarinic acetylcholine receptors (mAChRs).

Having established a protocol for evoking reproducible mAChR-mediated EPSPs ($EPSP_M$s) and mAChR-mediated inhibition of SFA, experiments were conducted to investigate the modulation of these responses by other neurotransmitter receptors. In this respect, activation of adenosine receptors by the broad spectrum adenosine receptor agonist 2-chloroadenosine (CADO) reversibly inhibited mAChR-mediated synaptic responses in a concentration dependent manner. The pharmacological profile of this effect established that it was mediated by adenosine A_1 receptors. The adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) alone facilitated cholinergic synaptic responses, demonstrating that these receptors are activated tonically *in vitro*. In support of this, 5-iodotubercidin, an adenosine kinase inhibitor which inhibits the uptake of adenosine, also caused a DPCPX-sensitive inhibition of $EPSP_M$s.

To assess whether CADO was acting pre- or post-synaptically to inhibit $EPSP_M$s, the concentration-response relationship for this effect was compared with relationships for known pre- and post-synaptic effects of CADO. The concentration-response relationship for the inhibition of $EPSP_M$s most closely resembled that for the presynaptic depression of glutamate receptor-mediated EPSPs, providing indirect evidence in favor of a presynaptic locus for the inhibition of $EPSP_M$s by CADO. That CADO was acting presynaptically was confirmed by the observation that

postsynaptic depolarization and inhibition of SFA evoked by carbachol (CCh) were not significantly affected by CADO at concentrations that significantly depressed cholinergic synaptic responses.

The effect of $GABA_B$ receptor activation on mAChR-mediated synaptic responses was also investigated. The selective $GABA_B$ receptor agonist baclofen (5 μM) inhibited $EPSP_M$s. However, at a concentration as high as 20 μM, baclofen had no consistent effect on the mAChR-mediated inhibition of SFA. The GABA uptake inhibitor NNC 05-0711 also inhibited $EPSP_M$s. The selective $GABA_B$ receptor antagonist CGP 55845A reversed the effects of both baclofen and NNC 05-0711. In addition, CGP 55845A, when applied alone, facilitated $EPSP_M$s. These data suggest that endogenously released GABA, acting at $GABA_B$ receptors, inhibits $EPSP_M$s and that this effect is enhanced by inhibition of GABA uptake.

In conclusion, it is possible to evoke isolated mAChR-mediated synaptic responses with a single stimulus in the CA1 region of the rat hippocampus. Adenosine A_1 receptors are present on cholinergic terminals in this region and act to inhibit these responses by an unknown mechanism. $GABA_B$ receptor activation also inhibits cholinergic synaptic transmission although the mechanism and site of action of this effect is unclear. Finally, endogenous adenosine and GABA, acting at adenosine A_1 and $GABA_B$ receptors respectively, inhibit cholinergic synaptic transmission *in vitro*. This modulation provides a potentially important mechanism for the control of neuronal excitability in the hippocampus *in vivo*.

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V. ABBREVIATIONS

(+)-MCPG	(+)-α-methyl-4-carboxyphenylglycine
(S)-DHPG	(*S*)-3,5-dihydroxyphenylglycine
3-APA	3-aminopropylphosphonic acid
4-AP	4-aminopyridine
4-CPG	(*S*)-4-carboxyphenylglycine
4-DAMP	*N*,*N*-dimethyl-4-piperidinyl diphenylacetate
5-IT	5-iodotubercidin
8-Br cAMP	8-bromoadenosine 3,5-cyclic monophosphate
8-PT	8-phenyltheophyline
A/D	analogue to digital
AD	Alzheimer's disease
A_1R	adenosine A_1 receptor
A_2R	adenosine A_2 receptor
A_3R	adenosine A_3 receptor
AC	adenylate cyclase
ACh	acetylcholine
AChE	acetylcholinesterase
ACSF	artificial cerebrospinal fluid
AD	Alzheimer's Disease
AFDX 116	11-2[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11,-dihydro-6H-pyrido[2,3b] [1,4]benzodiazepin-6-one
AHP	after hyperpolarization
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine 5'-triphosphate
ATPA	((*RS*)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl) propanoic acid)
CACA	*cis*-4-aminocrotonic acid
CADO	2-chloroadenosine
cAMP	cyclic adenosine 5'-monophosphate
CCh	carbachol
CCPA	2-chloro-N^6-cyclopentyladenosine
CGP 35348	*p*-[3-aminopropyl]-*p*-diethoxymethylphosphinic acid
CGP 40116	D-(*E*)-2-amino-4-methyl-5-phosphono-3-pentanoic acid
CGP 55845A	[1-(S)-3,4-dichlorophenyl)ethyl]amino-2-(S)-hydroxypropyl-*p*-benzyl-phosphonic acid
CGS 21680	2-*p*-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
D-AP5	D-2-amino-5-phosphonopentanoate
DC	direct current
DCG IV	(2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
DPY	dipyridamole
DRG	dorsal root ganglion

EAA	Excitatory Amino Acid
EPSP	excitatory postsynaptic potential
$EPSP_A$	AMPA receptor-mediated EPSP
$EPSP_M$	muscarinic acetylcholine receptor-mediated EPSP
$EPSP_N$	*N*-methyl-D-aspartate receptor-mediated EPSP
GABA	γ-aminobutanoic acid
G-protein	guanine nucleotide binding protein
GYKI 53655	(1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine)
I_{AHP}	calcium activated AHP current
$I_{K(LEAK)}$	leak K^+ current
IP_3	inositol 1,3,5-triphosphate
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
$IPSP_A$	$GABA_A$ receptor-mediated IPSP
$IPSP_B$	$GABA_B$ receptor-mediated IPSP
L-AP4	L-2-amino-4-phosphonobutanoic acid
LTP	long term potentiation
LY294486	((3SR, 4aRS, 6SR, 8aRS)-6-((((1H-tetrazol-5-yl)methyl)oxy) methyl)-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid)
mAChR	muscarinic acetylcholine receptor
MAP4	(*S*)-2-amino-2-methyl-4-phosphonobutanoic acid
MCCG	(2*S*,3*S*,4*S*)-2-methyl-2-(carboxycyclopropyl)glycine
nAChR	nicotinic acetylcholine receptor
NBQX	6-nitro-7-sulphamoylbenzo[*f*]quinoxaline-2,3-dione
NECA	5'-*N*-ethylcarboxamidoadenosine
NMDA	*N*-methyl-D-aspartate
NNC 05-0711	diphenylmethanone,0-[2-(3-carboxy-1,2,5,6-tetrahydro-1-pyridinyl)ethyl]oxime hydrochloride
PLC	phospholipase C
PLD	phospholipase D
PLA_2	phospholipase A_2
SCG	superior cercvical ganglion
QX 314	2-(triethyamonium)-*N*-(2,6-dimethylphenyl)acetamide
R-PIA	*R*(-)N^6-(2-phenylisopropyl) adenosine
s. oriens	*stratum oriens*
s. radiatum	*stratum radiatum*
s. pyramidali	*stratum pyramidali*
S.E.M.	standard error of the mean
SCG	superior cervical ganglion
SFA	spike frequency adaptation
THIP	4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol
V_m	membrane potential
ZM 241385	(4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,4]triazin-5-yl amino]ethyl) phenol)

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CHAPTER 1

GENERAL INTRODUCTION

The ability of the mammalian central nervous system (CNS) to function effectively as an information processor relies on the organization of neurones into circuits and networks. For these circuits to function, neurones must be able to communicate through the process of synaptic transmission. Synaptic transmission refers to the transmission of information across synapses through the release of neurotransmitters to act at postsynaptic receptors (see Shepherd & Koch, 1990). Acetylcholine (ACh) was the first compound to be identified as a neurotransmitter, initially at peripheral synapses (Dale, 1934) and subsequently in the CNS (Eccles *et al.* 1954; Eccles *et al.* 1956). The process of synaptic transmission itself can be modulated by receptor systems other than that which principally mediates synaptic transmission. Such neuromodulation can occur pre- and post-synaptically providing precise control of synaptic transmission and, as such, may be particularly important in controlling the transfer of information in neuronal circuits.

Although it is possible to mimic the effects of neurotransmitters pharmacologically, the only method by which to investigate the complete process of synaptic transmission is to study evoked synaptic responses mediated by endogenously released neurotransmitter. With respect to (acetyl)cholinergic synaptic transmission, synaptic responses may be evoked in hippocampal slices by electrical stimulation of the septohippocampal cholinergic input (Cole & Nicoll, 1983; 1984). The present study has utilized *in vitro* electrophysiological recording techniques to

i) establish an effective method with which to evoke and investigate the modulation of cholinergic synaptic responses and,

ii) investigate the influence of adenosine and GABA receptor activation on cholinergic synaptic transmission in the CA1 region of the rat hippocampus.

This chapter will serve as a general introduction to the major receptor systems and neurotransmitters dealt with in this thesis. Neurotransmitters ACh, glutamate and GABA, and the neuromodulator adenosine will be discussed in terms of their

receptor pharmacology and functional effects in the CNS. Where appropriate, aspects of neurotransmitter function will be discussed with particular reference to the hippocampus to maintain brevity and relevance to this study. In addition, the major functional and anatomical aspects of the hippocampus and relevant neuronal pathways will be introduced.

1.1. ACETYLCHOLINE

1.1.1. HISTORICAL PERSPECTIVES

During the early part of this century, a study of the action of an active component of adrenal extracts found that a derivative of choline reduced blood pressure in rabbits (Hunt & Taveau, 1906). This derivative was subsequently found to be ACh, providing the first evidence of a pharmacological action of ACh. Subsequently, a chance discovery by H. H. Dale (1914) led him to distinguish the nicotinic and muscarinic actions of ACh (Dale, 1914). However, it was a further seven years before the first firm evidence of chemical transmission by ACh was published by Loewi (Loewi, 1921). Loewi (1921) described the action of a substance released following vagal stimulation on isolated heart. This substance was later identified as ACh (Loewi & Navratil, 1926). During the 1930's these advances were further developed by Dale and coworkers when they published a series of papers proposing that ACh was a chemical transmitter in both the parasympathetic and sympathetic nervous systems (Dale, 1934; Dale *et al.* 1936).

Around this time, a neurotransmitter role for ACh in the CNS was postulated by Dale (1938) among others (Feldberg, 1945). This hypothesis was based on

i) the presence of ACh and acetylcholinesterase (AChE: an endogenous enzyme which breaks down extracellular ACh) in the CNS,

ii) the synthesis and release of ACh in the CNS, and

iii) the central effects of ACh and physostigmine (eserine) an inhibitor of AChE (Feldberg, 1945).

That cholinergic synapses existed in the CNS was not reported however until the 1950's, when Eccles and coworkers (1954; 1956) demonstrated the existence of a synapse operated by ACh between motor-axon collaterals and nicotinic ACh receptors (nAChRs) on Renshaw cells in the spinal cord. Since then classification of muscarinic ACh receptors (mAChRs) and nAChRs has been developed using pharmacological and molecular cloning techniques (Brann *et al.* 1993). As such, there are now multiple subtypes of both neuronal nAChRs and mAChRs (Hulme *et al.* 1990; Nicoll *et al.* 1992; Lindström *et al.* 1995a).

1.1.2. The Receptors and Their Pharmacology

The basic classification of ACh receptors is still based on Dale's original description of the nicotinic and muscarinic actions of ACh (Dale, 1914). The receptors that mediate muscarinic and nicotinic responses have entirely different structures and functions. Nicotinic ACh receptors are ligand (ACh) gated ion channel (ionotropic) receptors whereas muscarinic ACh receptors are guanine nucleotide binding (G)-protein coupled (metabotropic) receptors.

1.1.2.1. Muscarinic Acetylcholine Receptors

The metabotropic mAChR belongs to the gene superfamily of G-protein coupled receptors, which includes the β-adrenergic receptor, the adenosine receptor and rhodopsin (Hall, 1987). The structure of this group of receptor proteins is characterized by an extracellular N-terminal region, seven hydrophobic transmembrane spanning regions and an intracellular C-terminal region. Initial classification of a response as being muscarinic was based on antagonism of that response by atropine (Dale, 1914). Some 65 years later, further pharmacological investigation of mAChR-mediated effects led to the identification of two subtypes of muscarinic receptor (termed M_1 and M_2) based on the relative sensitivity of responses to pirenzipine (Hammer *et al.* 1980). The structural difference between these receptors has since been confirmed with the molecular cloning of both receptors (termed m1 and m2) (Kubo *et al.* 1986; Kubo *et al.* 1986). Three additional subtypes of muscarinic receptor have been identified by homology cloning. These have been termed m3, m4 and m5 (Bonner *et al.* 1987; Bonner *et al.* 1988).

Clear pharmacological classification of mAChR subtypes has been difficult to achieve. In this respect, compounds have been developed which exhibit limited selectivity for individual mAChRs, typically in the order of 10-fold selectivity for one subtype over another. Thus, pirenzipine, has been found to be relatively selective for the M_1 mAChR, and AFDX 116 to be relatively selective for M_2 mAChRs (Giachetti *et al.* 1986). In addition, the compound *N*,*N*-dimethyl-4-piperidinyl diphenylacetate (4-DAMP) is relatively selective for M_1 and M_3 mAChRs (Doods *et al.* 1987). Quantitative pharmacological investigations using these antagonists can be used to narrow down the identity of the subtype of mAChR but can prove inadequate to conclusively identify the exact subtype (Pitler & Alger, 1990; Mitchelson, 1988). As such, it is difficult to unequivocally identify mAChR subtypes due to the lack of specificity of the available antagonists and, in particular, the lack of potent and selective antagonists for M_4 and M_5 mAChRs. Indeed, in the hippocampus, there is some controversy regarding the subtype(s) of mAChRs which mediate the variety of mAChR-mediated effects evoked by either AChR agonists such as carbachol (CCh) and methacholine or by synaptically released ACh (Cole & Nicoll, 1984b; Benson *et al.* 1988; Dutar & Nicoll, 1988a; Pitler & Alger, 1990). A summary of the most subtype-specific mAChR antagonists presently available is provided in Table 1.1. Since few of these antagonists exhibit greater than 10-fold selectivity for one subtype over other mAChR subtypes, it is clear that more selective mAChR agonists and antagonists will be required to specifically inhibit the action of particular mAChRs.

The lack of mAChR subtype-specific ligands has also limited the amount of information, regarding the distribution of mAChR subtypes in the brain, that can be gained from binding and autoradiographic studies (Cortés & Palacios, 1986; Waelbroeck *et al.* 1990; Zubieta & Frey, 1993; Levey *et al.* 1994). However, such studies, in conjunction with *in situ* hybridization and immunocytochemical studies, have helped to provided information regarding the specific localization of mAChR subtypes in many areas of the CNS, including the hippocampus (Table 1.2; Buckley *et al.* 1988; Weiner & Brann, 1989; Levey *et al.* 1995).

Table 1.1. mAChR subtypes: Pharmacology and transduction mechanisms.

mAChR (molecular subtype)	M_1 (m1)	M_2 (m2)	M_3 (m3)	M_4 (m4)	M_5 (m5)
Selective Antagonists	Pirenzipine Telenzipine	Methoctramine Himbicine AFDX 116 Tripitramine	4-DAMP Darifenacin	Tropicamide Himbicine	None Known
G-protein Coupling	G_q/G_{11}	G_i/G_o	G_q/G_{11}	G_i/G_o	G_q/G_{11}
Signal Transduction Mechanisms	$\uparrow IP_3/DAG$	$\downarrow$cAMP	$\uparrow IP_3/DAG$	$\downarrow$cAMP	$\uparrow IP_3/DAG$
mAChR Protein/mRNA Expression in Hippocampus †	+++++	+++	++	+++	++
Expression of mAChR Proteins in the Hippocampus ‡	soma & dendrites of pyramidal neurones	nonpyramidal neurones and in fibers surrounding pyramidal neurones	pyramidal neurones	nonpyramidal neurones	no data

† (Brann *et al.* 1993); ‡(Levey *et al.* 1995).

All five subtypes of mAChR protein and mRNA have been localized in the hippocampus as well as the cortex, striatum, olfactory tubercle, thalamus and basal forebrain (Brann *et al.* 1993; Table 1.2.). In terms of the amount of expression of mAChR mRNA in the hippocampus as a whole, the rank order is m1>m2/m4>m3/m5 (Table 1.1). mAChR protein and mRNA have differential cellular distributions in different areas of the hippocampus, with m1 and m3 mAChR protein expression prominent in pyramidal neurones and m2 and m4 in non-pyramidal neurones and fiber pathways (Levey *et al.* 1995; Table 1.1). In addition, m2 mAChR proteins are expressed presynaptically in cholinergic axons presumably of the septo-hippocampal pathway (Levey *et al.* 1995; see also section 1.6.2). The

localization of m2 mRNA in the medial septum is consistent with this observation (Buckley *et al.* 1988; Rouse *et al.* 1997).

With respect to mAChR transduction mechanisms, it is clear that each mAChR subtype is coupled to G-proteins of either the G_i/G_o or G_q/G_{11} subtypes and, as such, mAChRs modulate a variety of signal transduction mechanisms (Table 1.1). In general, M_1, M_3 and M_5 mAChRs are positively linked, via the G_q/G_{11} subtype of G-protein, to the phospholipase C (PLC)/inositol triphosphate (IP_3) system and M_2 and M_4 mAChRs negatively linked, via the G_i/G_o subtype of G-protein, to the AC/cyclic adenosine 5' monophosphate (cAMP) system (Hulme *et al.* 1990; Felder, 1995).

Additional complexity to the functioning of mAChRs is offered by the presence of an allosteric binding site (Tucek & Proska, 1995). Neuromuscular blocking drugs such as gallamine allosterically inhibit the action of mAChR agonists at all five mAChR receptors, particularly the M_2 subtype (Ellis *et al.* 1991). In contrast, alcuronium can augment, as well as inhibit, the binding of mAChR ligands (Tucek *et al.* 1990).

1.1.2.2. Nicotinic Acetylcholine Receptors

In contrast to mAChRs, nAChRs belong to the gene superfamily of ion channel receptors, which includes $GABA_A$ and glycine receptors. nAChRs, which are activated by both ACh and nicotine, mediate synaptic transmission at both peripheral and central synapses. Responses mediated by nAChRs may be distinguished pharmacologically from mAChR-mediated responses using the competitive antagonist curare (Dale, 1914). nAChRs can now be broadly divided into,

i) muscle nAChRs,

ii) neuronal nAChRs that bind α-bungarotoxin, and

iii) neuronal nAChRs that do not bind α-bungarotoxin (see Lindström *et al.* 1995b).

Biochemical and molecular cloning techniques have revealed that these ACh-gated cation channels consist of a pentamer of homologous subunits, which assemble to form a central ion channel (McCarthy *et al.* 1986; Hucho *et al.* 1996). In addition to the subunits which form muscle and ganglionic nAChRs, eight neuronal α subunits

and three neuronal β subunits have been cloned (Albuquerque *et al.* 1997). These subunits form heterooligomeric complexes in which subunits combine with the stoichiometry 2α, 3β or homooligomeric complexes of α7 or α9 subunits. Each nAChR subunit composition exhibits different pharmacological and biophysical properties (Deneris *et al.* 1991; Alkondon & Albuquerque, 1993; McGehee & Role, 1995; Albequerque *et al.* 1995a). As such, α -subunits α2–α6 form distinct channels when expressed with different β-subunits in oocytes from *Xenopus laevis*. In contrast either α7-, α8- or α9-subunits alone, when expressed in oocytes, form homomers which support α-bungarotoxin-sensitive currents. Only α9 and, most prominently α7 subunits are found in the rat, whereas α8 subunits are only found in the chick (McGehee & Role, 1995). The rapidly desensitizing α7 nAChR is thought to be of particular functional importance due to its high permeability to Ca^{++} and its presence at presynaptic terminals.

The effective characterization of neuronal nAChRs has been restricted by the lack of selective ligands for the large variety of nAChR subunit constructs found in the CNS. In the hippocampus for example, nAChRs modulate at least three separate cationic currents which are termed types I–III (Alkondon & Albuquerque, 1993; Albuquerque *et al.* 1995a; Albuquerque *et al.* 1997). These currents may be identified by different channel kinetics and their sensitivity to nicotinic agonists and the nicotinic antagonists methylcaconitine, β-erythroidine, α-bungarotoxin and mecamylamine (Albuquerque *et al.* 1995b; Albuquerque *et al.* 1997). In the hippocampus α7 nAChRs are thought to underlie type 1A currents which are mediated predominantly by Ca^{++} (Albuquerque *et al.* 1995b). In addition to direct excitatory effects, nAChRs have also been shown to presynaptically modulate the release of neurotransmitters (e.g. glutamate and ACh) and glutamate synaptic transmission in several areas of the brain, including the hippocampus (e.g. Araujo *et al.* 1988; Gray *et al.* 1997, Wonnacott, 1997). The pharmacology, structure and function of nAChRs has been comprehensively reviewed (Sargent, 1993; Albuquerque *et al.* 1995b; Lindström *et al.* 1995a/b; McGehee & Role, 1995; Lindström *et al.* 1996; Wonnacott, 1997).

1.1.3. The Neurotransmitter Role of Acetylcholine

Both nAChRs and mAChRs have been shown to be synaptically activated in a number of well-defined cholinergic pathways in the mammalian brain (Eccles *et al.* 1954; Cole & Nicoll, 1983; Nicoll *et al.* 1992). The most well characterized of these is the septo-hippocampal cholinergic pathway, which innervates pyramidal neurones in the hippocampus (Dutar *et al.* 1995; Nicoll, 1985; See Section 1.6.2). Stimulation of this pathway, using high frequency stimulation, can evoke slow mAChR-mediated excitatory responses in CA1 pyramidal neurones (Cole & Nicoll, 1983, 1984a).

1.1.4. Muscarinic Acetylcholine Receptor-mediated Effects in the CNS

In contrast to the limited effects of nAChR activation in the CNS, mAChR activation can result in a variety of effects that are both excitatory and inhibitory (Brown *et al.* 1997). These effects include

i) inhibition/activation of certain K^+ conductances,

ii) inhibition/activation of voltage-dependent Ca^{++} currents, and

iii) activation of non-selective cationic currents.

In the hippocampus, the most extensively studied of these effects is the inhibition of K^+ conductances.

1.1.4.1. Effects on potassium channels

Synaptic activation of mAChRs in the hippocampus evokes a slow depolarization associated with an increase in input resistance (Cole & Nicoll, 1983; 1984), effects which are similar to those produced by application of CCh or ACh in, for example, hippocampus and cortex (see Table 1.2; Dodd *et al.* 1981; Haas, 1982; Segal, 1982; Cole & Nicoll, 1983, 1984a & 1984b). This depolarizing effect is due to an apparent inward current (Halliwell & Adams, 1982; Madison *et al.* 1987; Benson *et al.* 1988) which represents a decrease in a voltage-independent *leak K^+ current* ($I_{K(LEAK)}$) (Benardo & Prince, 1982b; Madison *et al.* 1987; Benson *et al.* 1988).

In addition to inhibition of $I_{K(LEAK)}$, activation of mAChRs leads to the inhibition of a

voltage independent and *Ca^{++} dependent K^+ current* ($I_{K(Ca)}$) known as the small-K current or I_{AHP} in a variety of brain regions (see Table 1.2; Madison & Nicoll, 1984; Constanti & Sim, 1987; Lancaster & Adams, 1986; Sah, 1996). The voltage- and Ca^{++}-dependent current (maxi-K current) I_C, however, is not affected by mAChR activation (Madison *et al.* 1987). In hippocampal pyramidal neurones, both I_{AHP} and I_C are activated by calcium entry during the depolarizing phase of an action potential. In this respect, the fast and slow afterhyperpolarizations (AHPs) which follow an action potential are mediated by I_C and I_{AHP}, respectively. During maintained depolarization of a neurone at firing threshold, the sAHPs which follow each successive action potential summate due to the slow inactivation of I_{AHP} (>1 s). This summation of the I_{AHP} results in a progressive increase in the duration between action potentials, a phenomenon known as spike frequency adaptation (SFA). As such, CCh has been shown to inhibit I_{AHP}, resulting in an inhibition of the slow AHP and hence inhibit SFA (Benardo & Prince, 1982a & 1982b; Cole & Nicoll, 1983; Madison *et al.* 1987; Dutar & Nicoll, 1988a). In addition, the cholinergic effects of CCh and ACh can be mimicked by stimulation of cholinergic afferents of the septo-hippocampal pathway (see section 1.6.2) in area CA1 of the hippocampus (Cole & Nicoll 1983, 1984; Nicoll, 1985). Thus, synaptically released ACh causes a depolarization and increase in input resistance, and inhibits slow AHPs and SFA (Cole & Nicoll, 1983, 1984b; Madison *et al.* 1987; Segal, 1988; Potier *et al.* 1992).

Other K^+ currents have been shown to be modulated by mAChRs, including the *M current* (I_M), a voltage-dependent K^+ current which does not inactivate with time. I_M was first described in bullfrog sympathetic neurones (Brown & Adams, 1980; Brown, 1983) and subsequently in hippocampal neurones (Halliwell & Adams, 1982) and was named M current because it is inhibited by muscarine, an mAChR agonist. Consistent with this, CCh and other mAChR agonists have been shown to inhibit I_M in the hippocampus (Halliwell & Adams, 1982; Brown, 1983; Madison *et al.* 1987; Benson *et al.* 1988; Dutar & Nicoll, 1988a; Halliwell, 1990), olfactory cortex (Constanti & Sim, 1987) and superior cervical ganglion (SCG; Marrion *et al.* 1989). Madison and co-workers (1987), showed that the concentration of CCh required to inhibit I_M was more than 10-fold higher than that required to inhibit I_{AHP} (Madison *et*

al. 1987), a similar observation has been made in the olfactory cortex (Constanti & Sim, 1987). Consistent with this, inhibition of I_M by synaptically released ACh in the hippocampus was not as readily achieved as inhibition of I_{AHP} or activation of the slow current underlying the slow EPSP (Madison *et al.* 1987). As such, it was concluded that inhibition of I_M did not contribute to the slow EPSP evoked by synaptically released ACh. In bullfrog sympathetic ganglion cells however, I_M is more readily inhibited than I_{AHP} by mAChR activation (Pennefather *et al.* 1985).

Other examples of mAChR-mediated modulation of K^+ conductances include the inhibition of the fast transient voltage-dependent *A current* (I_A) which normally enhances spike repolarization and slows discharge rate (Nakajima *et al.* 1986). In neostriatal neurones mAChR activation also inhibits I_A, which at hyperpolarized potentials tends to reduce action potential firing frequency, whereas at depolarized potentials mAChR activation inactivates I_A completely increasing neuronal excitability (Akins *et al.* 1990).

Conversely, mAChR activation in the hippocampus has also been shown to *activate* K^+ conductances such as the *delayed rectifier K^+ current* (I_K) (Zhang *et al.* 1992). In other brain areas, such as the thalamus, mAChRs have also been shown to cause a hyperpolarization associated with a conductance increase mediated by K^+ ions (e.g. McCormick & Prince, 1985). Finally, the *anomalous inward rectifying current* (I_Q), which is mediated by both K^+ and Na^+ ions, is also activated by mAChRs in the hippocampus (Colino & Halliwell, 1993).

1.1.4.2. Effects on calcium and nonselective cationic currents

Evidence for the effects of mAChR activation on Ca^{++} currents in the hippocampus is more limited. mAChR-mediated decreases in Ca^{++} currents have been reported in CA3 neurones in slice cultures (Gähwiler & Dreifuss 1982; Gähwiler & Brown 1987) and in the cortex and neostriatal neurones (Misgeld *et al.* 1986). mAChR activation has been shown to inhibit high voltage-activated Ca^{++} currents in rat embryonic hippocampal neurones (Toselli & Taglietti, 1995) and in excised membranes of hippocampal neurones (Toselli & Taglietti, 1994). In the superior cervical ganglion (SCG) and basal forebrain neurones mAChR-activation inhibits

Table 1.2. Summary of mAChR mediated effects and location in the mammalian brain.

	$I_{K(LEAK)}$	I_{AHP}	I_M	I_{Ca}	Other	mAChR binding	mAChR protein	mAChR mRNA	Cholinergic projection from→to
Cortex			↓	↓**(L)**		**++**	**+** (m1,m2,m4) §	**+++** (m3, m4) †	MS/DBB→ NBM→
Hippocampus	↓*	↓*	↓*	↓↑*	↓I_A ↑I_K	**++**	**+** (m1) §	**+** (m1, m2, m3, m4) † #	→MS/DBB→
Striatum	↓*			↓	↓I_A	**+**	**+** (m1<m4) §	**++**	Interneurones
Olfactory Bulb/Tubercle	↓	↓	↓			**+++**	**+** §	**++** (m1, m2)	DBB→
Thalamus	↓					**+** (non-M1) #	**+** (m2>m4) §	**+** (m3) †	DBB→
MS/DBB (Forebrain)				↓ (↑)*f*		**+** (non-M1)	**+** (m2, m3) §	**+** (m2) †	→Hippocampus, Cortex, Thalamus.
Brainstem						**+** (non-M1) #	**+** (m2) ‡ §	**+** (m3) †	
SN						**+** (M1) #		**+** (m5) ⊗	
SCG			↓	↓**(N)**					

KEY:

↓ inhibits
↑ activates
* Electrical stimulation mimics response
(N/L) N- or L-type Ca^{++} channels.
† Buckley *et al.* 1988
‡ Levey *et al.* 1994
# Palacios *et al.* 1990
f Reynolds & Miller, 1989
⊗ Brann *et al.* 1993
§ Cortés & Palacios, 1986
Mash & Potter, 1986
Spencer *et al.* 1986
Levey *et al.* 1994

N-type Ca^{++} channels (Plummer *et al.* 1991). However one study also suggests an inhibition of L-type channels in the SCG (Mathie *et al.* 1992), an effect also reported in the cerebral cortex (Boess *et al.* 1990).

A considerable amount of work has been carried out using neuroblastoma hybrid cells, transfected with cloned mAChR cDNA, to identify the mAChR subtypes mediating modulation of Ca^{++} currents (Brown *et al.* 1993). Using this approach m2 and m4 mAChRs but not m1 and m3 mAChRs have been found to inhibit a Ca^{++} current designated the "N" current (Higashida *et al.* 1990). This classification is somewhat misleading, as the kinetics differ from the classic N-type current. However, it is likely that this "N" current corresponds to Ca^{++} current(s) responsible for modulating transmitter release, i.e. N-type Ca^{++} channels. Inhibition of these currents is the likely mechanism for mAChR-mediated inhibition of transmitter release at presynaptic terminals (Wu & Saggau, 1997).

In contrast, Fraser & MacVicar (1991) have shown that mAChR stimulation activates a low-threshold transient (T-type) Ca^{++} current in hippocampal CA1 interneurones in the *laculosum-moleculare* region. The same group have also demonstrated a mAChR-mediated activation of a novel regenerating current, which underlies a plateau potential, that involves the interplay between a high-voltage-activated Ca^{++} conductance and a Ca^{++} activated nonselective cation conductance (Fraser & MacVicar, 1996).

mAChR-mediated depolarization through the activation of nonselective cationic conductances has also been described in CA1 and CA3 neurones in hippocampal slices and slice cultures, respectively (Colino & Halliwell, 1993; Caeser *et al.* 1993; Guérineau *et al.* 1995), as well as in cortical layer V pyramidal neurones (Haj-Dahmane & Andrade, 1996).

1.1.4.3. Functional consequences of mAChR activation

In the hippocampus, mAChR activation has been shown to inhibit glutamate and GABA release at synapses in the CA1 (Hounsgaard, 1978; Valentino & Dingledine, 1981; Dutar & Nicoll, 1988a; Cohen *et al.* 1991) and CA3 regions (Williams & Johnston, 1988). Transmitter release may be inhibited by

i) activation of K^+ conductances,

ii) a direct inhibition of the calcium-dependent release machinery, or

iii) the inhibition of Ca^{++} currents.

A recent paper, however, has suggested that mAChR-mediated presynaptic inhibition in area CA1 is mainly mediated by the inhibition of voltage-dependent Ca^{++} channels (Quian & Saggau, 1997).

Cholinergic action may also affect synaptic efficacy through modulation of synaptic plasticity, e.g. long term potentiation (LTP). In this respect activation of mAChRs enhances induction of LTP (Blitzer *et al.* 1990) and even induces a form of long term plasticity known as LTP_m (Auerbach & Segal, 1996). The role of mAChRs in memory has been reviewed in some detail (Krnjevic, 1993; Aigner, 1995; Segal & Auerbach, 1997).

Application of cholinergic agonists such as CCh can induce more complex effects such as theta (θ) rhythm-like activity (Konopacki *et al.* 1987; Bland *et al.* 1988). θ-like activity consists of membrane potential oscillations and rhythmic spike discharges at a frequency of 4–10 Hz and is thought to be functionally important in learning and memory. As such, disruption of the θ-rhythm in the rat impairs performance in spatial memory tasks (Winson, 1978). Related to this phenomenon is the mAChR-activated rhythmical slow activity (RSA), which has been recorded in CA3 hippocampal pyramidal neurones and consists of periodic bursts of 4–8 Hz oscillatory depolarizations (MacVicar & Tse, 1989; Traub *et al.* 1992).

In addition to the effects on the oscillatory behavior of hippocampal neurones, mAChR activation, using high concentrations of CCh, alters the action potential waveform by a Ca^{++} dependent mechanism (Figenschou *et al.* 1996). Clearly, therefore, the ability of mAChR activation to alter synaptic efficacy, oscillatory behavior and action potential waveform in the hippocampus indicates the importance of mAChR-mediated effects in network function in the hippocampus.

1.2. THE PURINES ADENOSINE AND ADENOSINE TRIPHOSPHATE

The purine nucleotide adenosine 5'-triphosphate (ATP) and nucleoside adenosine are unusual in that they are involved in cellular metabolic processes but also have actions at cell surface receptors (Williams, 1987). The receptors at which adenosine and ATP act are collectively known as purinoceptors (Burnstock, 1978). ATP, which acts at P_2 purinoceptors, is stored and released as a neurotransmitter in the peripheral and central nervous system (Edwards & Gibb, 1993; Fredholm *et al.* 1994b). In contrast, there is little evidence to suggest that adenosine, which acts at P_1 purinoceptors, acts as a classical neurotransmitter, however many cells synthesize and release adenosine (Stone *et al.* 1990). In addition, when ATP is released, it is rapidly broken down to adenosine by ectonucleotidases (Meghji, 1990). As such, adenosine is thought to act as a local hormone or neuromodulator and has many neuromodulatory roles, in the nervous system (Dunwiddie, 1985; Snyder, 1985; Daval *et al.* 1991). In the CNS, adenosine acts predominantly as an inhibitory modulator in that its main actions are reduction in cell firing or excitability and inhibition of transmitter release. A number of P_2 receptors have been pharmacologically identified and cloned, however the actions of ATP at this group of receptors are not relevant to this study. P_2 purinoceptors have been extensively reviewed in numerous recent publications (Fredholm *et al.* 1994a; Dalziel & Westfall, 1994; Kennedy & Leff, 1995; Fredholm *et al.* 1997).

1.2.1. EXTRACELLULAR ACTIONS OF ADENOSINE- AN HISTORICAL PERSPECTIVE

Drury and Szent-Györgyi in 1929, were the first to recognize the importance of adenosine as a physiologically active compound from studies on the mammalian heart. However, the primary evidence of the existence of receptors for adenosine came over 30 years later, with the observation that caffeine inhibited the action of adenosine on the mammalian heart (De Gubareff & Sleator, 1965). A rational system for the classification of purinoceptors was introduced in 1978, when Burnstock proposed a basis for distinguishing two types of purinergic receptor, namely adenosine (P_1) receptors and ATP (P_2) receptors (Burnstock, 1978). The following

year, the ability of adenosine to both inhibit and stimulate adenylate cyclase (AC) activity led to the subclassification of adenosine receptors into adenosine A_1 and A_2 receptors (A_1Rs and A_2Rs respectively) (Sattin & Rall, 1970; Van Calker *et al.* 1979; Londos *et al.* 1980). This classification system also relied on the agonist potency ratios of adenosine and its related analogues. With the subsequent development of more selective agonists and antagonists, it is now relatively easy to identify the actions of each subtype of adenosine receptor using subtype specific agonists and antagonists (Collis & Hourani, 1993; Dalziel & Westfall, 1994). More recently, the use of molecular biological techniques has led to the proposal of a third subgroup of adenosine receptors termed A_3 receptors (Caruthers & Fozard, 1993; Collis & Hourani, 1993). The current system of classification of adenosine receptors is summarized in Table 1.3.

Table 1.3. Adenosine receptor subtypes: Pharmacology and transduction mechanisms.

Subtype	A_1	A_{2a}	A_{2b}	A_3
Selective agonists	CCPA	CGS 21680	none known (NECA)	APNEA
Agonist Potency Ratios	R-PIA>NECA >S-PIA	NECA>R-PIA >S-PIA	NECA>R-PIA >S-PIA	NECA=R-PIA >S-PIA
Antagonists	DPCPX	ZM 241385	none known	I-ABOPX
Signal Transduction Mechanisms	↓AC	↑AC	↑AC	↓AC
G-protein Interaction	G_i/G_o	G_s	G_s	?

Abbreviations: APNEA: N^6-2-(4-amino-3-iodophenyl)-ethyladenosine.

1.2.2. ADENOSINE RECEPTORS

Adenosine receptors, like mAChRs are members of the G-protein coupled receptor superfamily whose structures are characterized by seven transmembrane hydrophobic domains (Olah & Stiles, 1992). Adenosine receptors can be found on a variety of peripheral tissues and in the CNS and are located both pre- and post-synaptically (Thompson *et al.* 1992). Historically, it was the observation that adenosine could

modulate AC that led to the increased interest in the actions of adenosine at cell surface receptors (Sattin & Rall, 1970). In addition to the modulation of AC, adenosine receptors may also couple to phospholipase C (PLC) and phospholipase A_2 (PLA_2), guanylyl cyclase and several K^+ and Ca^{++} channels (Olsson & Pearson, 1990) to mediate a variety of functions both in peripheral and neuronal tissues. Four main adenosine receptor subtypes (A_1, A_{2a}, A_{2b} and A_3) have been proposed and are now generally accepted on the basis of pharmacological profiles, second messenger systems and receptor structure (Abbaracchio *et al.* 1993).

1.2.2.1. Adenosine A_1 Receptors

A_1Rs are negatively linked to AC and so inhibit the turnover of intracellular cAMP. This observation, and the rank order of potency of adenosine and its analogues N^6-phenylisopropyladenosine (PIA) and 5'-*N*-ethylcarboxamideadenosine (NECA), was the basis for the initial classification of A_1Rs. Thus, the rank order of agonist potency of these agonists at A_1Rs was proposed to be PIA > adenosine > NECA (Londos *et al.* 1980). At present there are a number of other analogues of adenosine which are useful in the investigation of the effects of adenosine receptor activation. 2-chloroadenosine (CADO), is an analogue of adenosine which is resistant to the actions of adenosine deaminase (which breaks down adenosine to inosine causing its inactivation). As such, CADO is a useful tool where the breakdown of adenosine will adversely affect its apparent potency. CADO is only slightly more selective for A_1Rs over A_2Rs, however, a stereoisomer of PIA, R-PIA, is relatively subtype selective for A_1Rs. The most useful tool for the investigation of A_1R-mediated effects is the agonist 2-chloro-N^6-cyclopentyladenosine (CCPA) which is a highly selective agonist at these receptors (Lohse *et al.* 1988). In terms of classification of A_1Rs using antagonists, early studies relied on antagonists such as 8-phenyltheophyline (8-PT), which has antagonist activity at both A_1Rs and A_2Rs. However, the development of xanthine derivatives such as the selective A_1R antagonist DPCPX has led to more reliable classification of A_1R-mediated effects. In this respect, DPCPX has a 700-fold selectivity for A_1Rs over A_2Rs (Lohse *et al.* 1987).

1.2.2.2. Adenosine A_2 Receptors

Adenosine A_2 receptors were initially separated from A_1Rs on the basis of their ability to stimulate, rather than inhibit, AC activity (Van Calker *et al.* 1979; Londos *et al.* 1980). In addition, the rank order of agonist potency for A_2 receptors was proposed to be NECA > PIA ≥ adenosine. More recently, however, A_2Rs were subdivided into two subtypes, namely a high affinity receptor (A_{2a}R) and a low affinity receptor (A_{2b}R), both of which are positively coupled to AC (Daly *et al.* 1983; Bruns *et al.* 1986; Ongini & Fredholm, 1996). Pharmacological classification of A_{2a}Rs and A_{2b}Rs was made easier in 1989, when CGS 21680 was first described as a potent and selective adenosine A_{2a} receptor agonist with 140-fold selectivity over A_1Rs (Jarvis *et al.* 1989). More recently a potent and A_{2a}R selective non-xanthine antagonist ZM 241385 (4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,4]triazin-5-yl amino]ethyl) phenol) has also been developed (Poucher *et al.* 1995).

1.2.2.3. Adenosine A_3 Receptors

The proposal of an A_3 receptor subtype has arisen from the cloning and functional characterization of a novel adenosine receptor from a cDNA sequence from the rat striatum (Zhou *et al.* 1992). When stably expressed in Chinese hamster ovary cells adenosine receptor agonists bound to this receptor with the potency order R-PIA = NECA > S-PIA. This order of agonist potency does not correlate with that for either A_1Rs or A_2Rs and, in addition, the receptor is resistant to blockade by certain xanthine antagonists. However, many of the compounds used in the study of A_3R-mediated effects also have activity at A_1Rs and, like A_1Rs, A_3Rs are negatively coupled to AC (Zhou *et al.* 1992).

1.2.3. The Neuromodulatory Role of Adenosine

Unlike ATP, which is released from purinergic nerves (Burnstock, 1972), there is little evidence to support a neurotransmitter role for adenosine (Bruns, 1991). Adenosine receptors, however, are widely distributed in the periphery and in the CNS and adenosine is released from neurones (Bender *et al.* 1981; Jonzon & Fredholm, 1985; Stone *et al.* 1990; Mitchell *et al.* 1993; Fredholm *et al.* 1994b). As

such, adenosine is thought to have a neuromodulatory role in the CNS.

Adenosine has many actions, mainly inhibitory, in the CNS. The principle action of adenosine is inhibition of transmitter release at a variety of excitatory and inhibitory synapses (Dunwiddie, 1990). Adenosine also has direct postsynaptic effects, such as, modulation of postsynaptic K^+ or Ca^{++} conductances and inhibition of spontaneous neuronal firing (see section 1.2.4). These actions alone provide strong evidence for a neuromodulatory role for adenosine. In support of this, the excitatory action of caffeine and xanthine adenosine receptor antagonists alone in neuronal systems both *in vitro* and *in vivo* suggests that adenosine tonically activates adenosine receptors (Greene *et al.* 1985; Bauman *et al.* 1992; Kaplan *et al.* 1993; Dunwiddie & Diao, 1994). This so called "purinergic inhibitory tone" (Harms *et al.* 1978) is enhanced by the inhibition of adenosine breakdown or uptake (Jackisch *et al.* 1984; Pak *et al.* 1994; Dunwiddie & Diao, 1994).

Although the extracellular actions of adenosine are well established, the source of extracellular adenosine is controversial. Adenosine is a major component of cellular metabolic processes (Arch & Newsholme, 1978) and, as such, it is difficult to pinpoint localized production of adenosine. Electrical stimulation and K^+-induced depolarization evokes adenosine release, however this process is thought to be carrier mediated as opposed to an exocytotic process (Sweeney, 1996). Another major source of adenosine is 5'-nucleosidase mediated breakdown of ATP. Extracellular concentrations of adenosine are highly dependent on the metabolic state of the surrounding neurones. As such, during hypoxia, concentrations of adenosine are dramatically elevated (Hagberg *et al.* 1987; Rudolphi *et al.* 1992). In this respect, due to its inhibitory role in the CNS and vasodilatory role in cardiac tissues, adenosine is thought of as a neuro- or cardio-protective agent. A more general description came from Newby (1984), who described adenosine as a "retaliatory metabolite".

1.2.4. Adenosine Receptor-mediated Effects in the CNS

The effects of adenosine receptor activation in the CNS can be separated into three general categories

i) inhibition of spontaneous neuronal firing,

ii) modulation of K^+ or Ca^{++} conductances, and

iii) presynaptic modulation of neurotransmitter release.

Each of these effects have been extensively characterized in the hippocampus.

1.2.4.1. The Effects of Adenosine on Spontaneous Neuronal Firing

Adenosine receptor activation has been shown to inhibit spontaneous neuronal firing and epileptic bursting (Phillis *et al.* 1979; Dunwiddie, 1980; Lee *et al.* 1984; Schubert & Lee, 1986). These effects are mediated by postsynaptic A_1Rs, as they are inhibited by A_1R antagonists and can be observed in the absence of synaptically released transmitters. As such, these effects are likely to be due to the activation of a postsynaptic K^+ conductances which result in hyperpolarization and enhancement of I_{AHP} (see section 1.2.4.2) thus reducing postsynaptic excitability.

1.2.4.2. The Effects of Adenosine on K^+ and Ca^{++} Channels

In the hippocampus, adenosine receptor activation has been shown to activate an voltage insensitive K^+ conductance, which mediates a hyperpolarization associated with a decrease in input resistance (Okada & Ozawa, 1980; Segal, 1982; Greene & Haas, 1985). This effect is mediated by a pertussis toxin sensitive G-protein (G_i or G_o) but does not involve the second messenger cAMP (Trussel & Jackson 1987). In addition, adenosine receptor activation also enhances I_{AHP}, which underlies the slow afterhyperpolarisation (slow AHP) in hippocampal pyramidal neurones (Haas & Greene 1984). One study has even reported that, in cultured hippocampal neurones, activation of adenosine receptors activates a voltage-dependent Cl^- conductance (Mager *et al.* 1990).

There is some evidence for adenosine receptor-mediated inhibition of Ca^{++} currents, both pre- and post-synaptically, in the CNS. In the hippocampus, adenosine inhibits regenerative Ca^{++}-dependent action potentials (calcium spikes) although it is not clear if this effect is direct or whether it is via activation of a K^+ conductance which subsequently inhibits a Ca^{++} conductance (Proctor & Dunwiddie 1983). In addition, adenosine receptor activation has been shown to inhibit N-type Ca^{++} currents in

hippocampal CA3 neurones (Mogul *et al.* 1993) and mouse motoneurones (Mynlieff & Beam, 1994). In cultured DRG neurones, adenosine receptor activation is reported to inhibit the duration of Ca^{++} spikes (Dolphin *et al.* 1986; Macdonald *et al.* 1986).

1.2.4.3. Modulation of Transmitter Release by Adenosine

As discussed already, adenosine is not thought to act as a neurotransmitter but as a neuromodulator (see section 1.2.3). Inhibition of transmitter release is perhaps the best-recognized neuromodulatory effect of adenosine receptor activation. The earliest reports of an inhibition of ACh release came from studies at the neuromuscular junction (Ginsborg & Hirst, 1972). In terms of neurochemical release, activation of presynaptic adenosine receptors has been shown to inhibit the release of excitatory and inhibitory neurotransmitters, such as, ACh, adrenaline, dopamine, GABA, glutamate and serotonin (Harms *et al.* 1978; Harms *et al.* 1979; Burke & Nadler, 1988). The inhibition of GABA release, however, is not a consistent finding in all brain regions as, for example, adenosine inhibits GABA release in the cortex and striatum but not the hippocampus (Harms *et* al. 1979; Hollins & Stone, 1980).

Although there is a substantial amount of evidence for the presynaptic modulation of transmitter release, the mechanism for these effects is controversial. Three possible hypotheses have been proposed on the basis of the known effects of adenosine:

i) adenosine activates a presynaptic K^+ conductance, resulting in a reduction in Ca^{++} influx, leading to a reduction in transmitter release,

ii) adenosine inhibits a presynaptic Ca^{++} conductance, also leading to a reduction in transmitter release, or

iii) adenosine acts directly on the Ca^{++} sensitivity of the release process to inhibit transmitter release (Silinsky, 1986)

With respect to the inhibition of presynaptic Ca^{++} conductances, adenosine has been shown to inhibit presynaptic Ca^{++} flux measured using Ca^{++}-imaging techniques in the guinea pig hippocampus (Wu & Saggau, 1994) and chick ciliary ganglion (Yawo & Chuhma, 1993). In the hippocampus this inhibition is thought to involve ω-

conotoxin GVIA-sensitive N-type and possibly Q-type Ca^{++} channels (Wu & Saggau, 1994). In other brain areas such as the rat brainstem, adenosine, acting at A_1Rs, has been shown to inhibit synaptic transmission predominantly via inhibition of an N-type Ca^{++} channel (Umemiya & Berger, 1994). In contrast, there is some evidence that activation of adenosine A_2Rs potentiates P-type Ca^{++} currents in hippocampal CA3 neurones and rat brainstem thereby facilitating synaptic transmission in the brainstem (Mogul *et al.* 1993; Umemiya & Berger, 1994).

1.3. THE INHIBITORY AMINO ACID GABA

1.3.1. GABA – AN HISTORICAL PERSPECTIVE

Decarboxylation of L-glutamate at its α-carbon atom (a process that is intrinsic to the natural breakdown of L-glutamate) yields the now widely accepted inhibitory neurotransmitter, GABA. The inhibitory properties of this neutral amino acid were first suggested by the demonstration that extracts of mammalian brain, containing GABA (Florey, 1961; Dudel *et al.* 1963), inhibited the firing of the crayfish stretch receptor in an identical manner to the purified compound (Kuffler & Edwards, 1958; Kravitz *et al.* 1963a; Kravitz *et al.* 1963b). GABA has since satisfied all the criteria required for its classification as a neurotransmitter (Kelly & Beart, 1975; Bowery & Nistico, 1989; Sivilotti & Nistri, 1990; Florey, 1991). In particular, the inhibitory role of GABA in the CNS was confirmed by the similarity of the Cl^- ion dependency and reversal potentials of inhibitory postsynaptic potentials (IPSPs) and GABA-induced membrane hyperpolarizing responses in the cerebral cortex (Krnjevic & Schwartz, 1967).

1.3.2. GABA RECEPTORS

1.3.2.1. Historical Perspectives

Historically, it was thought that both pre- and post-synaptic actions of GABA were mediated via a single receptor which universally gated a Cl^- conductance (Coombs *et al.* 1955; Krnjevic, 1974; Eccles *et al.* 1977; Allen *et al.* 1977; Sakmann *et al.* 1983). This receptor was selectively blocked by the plant alkaloid bicuculline (Curtis *et al.*

1970a) and was later designated the $GABA_A$ receptor (Hill & Bowery, 1981). Since then $GABA_A$ receptors have been described in nearly every region of the CNS where classically they mediate a fast hyperpolarizing IPSP (Bormann, 1988; Stephenson & Dolphin, 1989; Sivilotti & Nistri, 1990).

Investigation of presynaptic GABA-mediated inhibition in rat post-ganglionic sympathetic nervous tissue using baclofen, the β-*p*-chlorophenyl- analogue of GABA (Bowery, 1982), led to the discovery of a second type of GABA receptor (Bowery *et al.* 1980). This receptor was insensitive to bicuculline and was later termed the $GABA_B$ receptor (Hill & Bowery, 1981). Unlike $GABA_A$ receptors, $GABA_B$ receptors are coupled through G-proteins to both ionotropic and metabotropic responses (Bormann, 1988; Bowery, 1989; Wojcik *et al.* 1990; Crawford & Young, 1990). These effects, like those mediated by $GABA_A$ receptors, occur at both pre- and post-synaptic loci (Dutar & Nicoll, 1988b; Deisz & Zieglgansberger, 1990; Waldmeier & Baumann, 1990; Dolphin *et al.* 1990). The regional distribution of $GABA_A$ and $GABA_B$ receptors is extremely similar (Bowery *et al.* 1984; Young & Chu, 1990). Nevertheless, the relative densities of both GABA receptor-subtypes varies in different brain regions (e.g. in the cerebellum, $GABA_A$ receptors predominate in the granule cell layer and $GABA_B$ receptors in the interpeduncular nucleus (Hill & Bowery, 1981)).

A third GABA receptor has now been identified, the so-called $GABA_C$ receptor. Like classical $GABA_A$ receptors, $GABA_C$ receptors are ligand-gated Cl^- channels but unlike $GABA_A$ receptors they are insensitive to bicuculline and to allosteric modulation by benzodiazepine and barbiturate site ligands. Although the $GABA_B$ receptor agonist (-)-baclofen is inactive at $GABA_C$ receptors, *cis*-4-aminocrotonic acid (CACA) selectively activates these receptors (Polenzani *et al.* 1991). Structurally, $GABA_C$ receptors are thought to be composed of the molecularly identifiable ρ1 and ρ2 subunits, which are expressed significantly in the rat retina (ρ1), hippocampus and cortex (ρ2; Enz *et al.* 1995). $GABA_C$ receptor-like effects have been observed in the hippocampus (Strata & Cherubini, 1994), although there is no firm evidence for their synaptic activation.

1.3.2.2. $GABA_A$ Receptors

The $GABA_A$ receptor is closely related to the same ligand-gated channel superfamily as nAChRs (Barnard *et al.* 1987; Schofield *et al.* 1987). Like many other receptors in this superfamily, multiple isoforms of $GABA_A$ receptors are likely to exist *in situ* (Schofield *et al.* 1987; Levitan *et al.* 1988; Olsen & Tobin, 1990; Tobin *et al.* 1991). In this respect six α-, four β-, three γ-, one δ- and one ε-subunit have now been identified using molecular cloning techniques. These subunits are thought to form heterooligomeric receptors with the stoichiometry $(\alpha)_2\beta(\gamma)_2$ where the γ-subunit can be replaced by a δ- or ε-subunit. (Sieghart, 1995).

The $GABA_A$ receptor is a multi-ligand binding complex (Simmonds, 1983; Bowery, 1984) which, in addition to the classical competitive agonist and antagonist binding site, includes sites for barbiturates, benzodiazepines, neurosteroids and ethanol (for review see Sieghart, 1995). Pharmacological activation of $GABA_A$ receptors can be achieved using 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) and muscimol which are two of the most potent and selective $GABA_A$ receptor agonists (Krogsgaard-Larsen *et al.* 1977). In addition, bicuculline or picrotoxin are often used to competitively and non-competitively antagonize $GABA_A$ receptors, respectively (Simmonds, 1980). Two different $GABA_A$ receptor-mediated responses have been reported to occur following afferent stimulation within a single neurone type i.e. "synaptic" (hyperpolarizing) and "extra-synaptic" (depolarizing) responses (Barker & Ransom, 1978; Thalmann *et al.* 1981; Alger & Nicoll, 1982a).

1.3.2.3. $GABA_B$ Receptors.

In contrast to $GABA_A$ receptors, $GABA_B$ receptors belong to the G-protein linked superfamily of receptors (Kuriyama & Ohmori, 1990) and have only recently been cloned (Kaupmann *et al.* 1997). To date, the most widely used selective $GABA_B$ receptor agonist is the (-)-isomer of baclofen (Bowery, 1982). 3-aminopropylphosphinic acid (3-APA) has been reported to be approximately 10–20 times more potent than (-)-baclofen (Harrison *et al.* 1990), however, under certain conditions, it may exhibit partial agonist properties (Bowery, 1989).

The first competitive $GABA_B$ receptor antagonist to be developed was a phosphinic acid derivative of baclofen, for which the colloquial name phaclofen was assigned (Kerr *et al.* 1987; Kerr *et al.* 1989b). This antagonist has since been superseded by sulphinic analogues, such as 2-hydroxy-saclofen, which have a 10-fold higher antagonist potency (Kerr *et al.* 1988; Kerr *et al.* 1989a). These compounds are still relatively weak and exhibit some loss of selectivity at the high (mM) concentrations required to block $GABA_B$ receptor-mediated synaptic responses (Davies *et al.* 1990; Mott & Lewis, 1991). The first potent (equipotent to 2-hydroxy-saclofen) and selective $GABA_B$ receptor antagonist to be developed was the *N*-substituted phosphinic analogue of GABA, *P*-[3-aminopropyl]-*P*-(diethoxymethyl)phosphinic acid, commonly referred to as CGP 35348 (Olpe *et al.* 1990). More recently, however, the selective $GABA_B$ receptor antagonist CGP 55845A (3-*N*- [1-(*S*)-(3,4-dichlorophenyl)ethyl] amino-2-(S)-hydroxypropyl-*P*-benzyl-phosphinic acid), has been shown to be 500–1000 more potent than CGP 35348 (Davies *et al.* 1993; Froestl *et al.* 1995). In addition, non-competitive antagonism of $GABA_B$ receptor-mediated responses also provides a powerful tool with which to probe $GABA_B$ receptor function. This can be attained for certain $GABA_B$ receptor-mediated responses by means of intracellular application of Cs^+ (Randall *et al.* 1991; Yoon & Rothman, 1991) or QX 314 (2-(triethylaminium)-*N*-(2,6-dimethylphenyl)acetamide) (Nathan *et al.* 1990) which are thought to block the $GABA_B$ receptor-operated K^+ conductance.

1.3.3. $GABA_B$ Receptor-mediated Effects in the CNS

The susceptibility of certain $GABA_B$ receptor-mediated effects to pertussis toxin suggests that $GABA_B$ receptors are coupled to ion channels through the G_i or G_o type of G-protein (Andrade *et al.* 1986; Thalmann, 1988; Bormann, 1988). Indeed, the classic $GABA_B$ receptor-mediated late or slow IPSP, seen throughout the CNS, is generated by the coupling of this type of G-protein to a "promiscuous" inwardly rectifying K^+ conductance that is thought to be shared by numerous other receptor systems including serotonin $5\text{-}HT_{1A}$ receptors (Andrade *et al.* 1986) and M_2 mAChRs (Christie & North, 1988). Similarly, $GABA_B$ receptor-mediated inhibition of

voltage-dependent Ca^{++} currents and the consequent reduction in the Ca^{++}-dependent plateau phase of the action potential (Dunlap & Fischbach, 1981) can be accounted for by direct inhibition of a Ca^{++} conductance by a pertussis toxin-sensitive $G\alpha_o$-protein (Deisz & Lux, 1985; Dolphin & Scott, 1987; Dolphin *et al.* 1989; Campbell *et al.* 1993). This mechanism is thought to account for $GABA_B$ receptor-mediated presynaptic inhibition of transmitter release and consequent depression of synaptic transmission. However, presynaptic inhibition by $GABA_B$ receptors in the hippocampus is not sensitive to pertussis toxin (Dutar & Nicoll, 1988b; Harrison, 1990). In addition, $GABA_B$ receptors do not affect the amplitude or transmission of the presynaptic Na^+-dependent action potential (Peng & Frank, 1989) but may modulate presynaptic K^+ conductances such as the fast activating I_A (Saint *et al.* 1990).

In addition to these effects, $GABA_B$ receptors, through their activation of G-proteins, can also invoke multiple intracellular biochemical effects, including inhibition of AC and enhancement of IP_3 production (Karbon *et al.* 1990; Wojcik *et al.* 1990; Crawford & Young, 1990). These complex intracellular effects are likely to explain the indirect $GABA_B$ receptor-mediated promotion and/or inhibition of both cyclic adenosine 5'-monophosphate (cAMP) and inositide formation induced by other agents e.g. noradrenaline and histamine.

1.4. THE EXCITATORY AMINO ACIDS GLUTAMATE AND ASPARTATE

1.4.1. EXCITATORY AMINO ACIDS – AN HISTORICAL PERSPECTIVE

The increased neuronal activity and susceptibility to epileptic seizures that followed *in vivo* CNS application of L-aspartate and L-glutamate first suggested that these endogenous acidic amino acids fulfill an excitatory role in the CNS (Hayashi, 1952; Hayashi, 1954). Indeed, intracellular recording subsequently revealed that they strongly depolarized both excitatory and inhibitory neurones (Curtis *et al.* 1959; Curtis *et al.* 1960; Curtis *et al.* 1961). The concept that specific receptors mediated this depolarization was confirmed by the strict structural requirements for compounds to evoke this response (Curtis & Watkins, 1960; Curtis & Watkins,

1963). Indeed, quantitative structure-activity relationships for L-glutamate and L-aspartate indicated that these amino acids acted at independent receptor sites (McLennan *et al.* 1968; Duggan, 1974). In particular, C-fiber compound action potentials were insensitive to L-aspartate but were readily suppressed by L-glutamate (Davies *et al.* 1979b; Evans, 1980; Agrawal & Evans, 1986). Within a few years, it was accepted that three pharmacologically distinct excitatory amino acid (EAA) receptor subtypes existed. These three subtypes were defined by the agonists *N*-methyl-D-aspartate (NMDA), kainate and quisqualate (Johnston *et al.* 1974; McCulloch *et al.* 1974; Watkins & Evans, 1981). However, more recently, the development of more selective pharmacological agents has led to the reclassification of EAA receptors into four broad subtypes (Watkins *et al.* 1991; Lodge & Johnson, 1991; Young & Fagg, 1991).

1.4.2. Excitatory Amino Acid Receptors

The most well studied of the EAA receptor subtypes are the so-called α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA; formerly known as quisqualate receptors) and NMDA receptors. These ionotropic receptors are believed to mediate the faster and slower components of dual component EPSPs evoked in the hippocampus, respectively (Dale & Roberts, 1985; Bekkers & Stevens, 1989; Headley & Grillner, 1991). Such EPSPs are characteristic of fast excitatory synaptic transmission throughout the vertebrate CNS. An additional "non-NMDA" ionotropic receptor subtype exists, namely the kainate receptor, which exhibits anomalous regional distribution to AMPA receptors. This receptor subtype also mediates synaptic transmission but only under conditions where repetitive afferent stimulation is used (Vignes & Collingridge, 1997).

The final EAA receptor subtype is commonly referred to as the metabotropic class of receptor of which nine different molecular entities have so far been isolated. These metabotropic glutamate receptors differ from the ionotropic receptor subtypes in that they directly activate numerous intracellular biochemical cascades through their coupling with both pertussis toxin sensitive and insensitive G-proteins (Schoepp *et al.* 1991).

1.4.2.1. NMDA Receptors

Since the molecular cloning of the NMDA NR1 subunit was achieved in 1991 (Moriyoshi *et al.* 1991) a further four NR2 subunits in the rat (NR2A, NR2B, NR2C and NR2D) have been cloned (Sucher *et al.* 1996). NR1 subunits can form homomeric complexes whereas NR1 and NR2 subunits form heteromeric structures of unknown stoichiometry (Sucher *et al.* 1996). As with nAChRs, different subunit assemblies posses different pharmacological and biophysical properties.

NMDA ion channel receptors bind both glutamate and NMDA, although activation requires glycine to bind as a co-agonist (Johnson & Ascher, 1987). One of the first selective and competitive antagonists to be developed for any one of the excitatory amino acid receptors was D-2-amino-5-phosphonopentanoate (D-AP5), which is selective for the NMDA receptor (Davies *et al.* 1981; Evans *et al.* 1982). Significantly, electrophysiological studies using this antagonist demonstrated that NMDA receptors could be activated physiologically in the CNS (Herron *et al.* 1986; Ascher & Nowak, 1987). In particular, in the CA1 region of the hippocampus, the ease with which it is possible to evoke NMDA receptor-mediated responses (Collingridge *et al.* 1988a; Collingridge *et al.* 1988b) correlates well with the extraordinarily dense NMDA receptor binding observed in this brain region (Monaghan & Cotman, 1985).

Non-competitive antagonism of NMDA receptor-mediated responses was first shown for Mg^{++} (Evans *et al.* 1977; Davies *et al.* 1979a; Mayer & Westbrook, 1987; Ascher & Nowak, 1988). At physiological concentrations, this divalent cation blocks the NMDA receptor-operated channel. In this respect, although this cation can enter the NMDA receptor-operated channel, its large size prevents free passage through the channel. Consequently, it prevents the normal passage of Na^{+}, K^{+} and Ca^{++}, which mediate the NMDA receptor-mediated synaptic current (Sucher *et al.* 1996). In addition several pharmacological compounds, such as MK 801, have been developed which, like Mg^{++}, non-competitively antagonize NMDA receptors.

1.4.2.2. AMPA Receptors.

The regional distribution of AMPA receptors within the CNS closely correlates with that of NMDA receptors (Bekkers & Stevens, 1989; Young & Fagg, 1991) and supports the concept that dual EPSPs in the brain are mediated by these two EAA receptors. Somewhat unusually, the amino acid sequences and overall structural characteristics of both these receptors differ from those of other ionotropic superfamily receptors in that each subunit is proposed to have only three transmembrane domains as opposed to the four proposed for the nAChR (McCarthy *et al.* 1986; Barnard & Henley, 1991). Their pharmacological separation was first achieved using D-AP5 to block NMDA receptor-mediated EPSPs ($EPSP_N$) (Collingridge *et al.* 1983). However, with the development of the quinoxalenedione family of non-NMDA receptor antagonists, such as 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Honore, *et al.* 1988; Blake *et al.* 1989), it has been possible to convincingly isolate intact $EPSP_N$s (Andreasen *et al.* 1988; Blake *et al.* 1988; Davies & Collingridge, 1989). A more potent non-NMDA receptor antagonist is NBQX, which has been shown to inhibit AMPA/kainate receptor-mediated EPSPs in the hippocampal area CA1 (Randle *et al.* 1992). More recently, GYKI53655, which is selective for AMPA over kainate receptors, has been described (Paternain *et al.* 1995).

1.4.2.3 Kainate Receptors

The kainate receptors have a structure which conforms more closely with classical ion channel receptors such as the nAChR (Hollmann & Heinemann, 1994). Three low affinity (GluR5-7) and two high affinity and pharmacologically distinct (KA1-KA2) kainate receptors have been cloned. Kainate receptors are widely distributed throughout the brain but are particularly abundant in area CA3 of the hippocampus and in spinal cord C-fibers (Pook *et al.* 1993). The demonstration of functional kainate receptors in the brain has been aided by the introduction of the highly selective AMPA receptor antagonist GYKI 53655 (1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine), allowing the investigation of pure kainate receptor mediated effects (Paternain *et al.*

1995). In addition, development of more selective ligands ATPA ((*RS*)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl)propanoic acid), a GluR5 agonist and LY294486 ((3SR, 4aRS, 6SR, 8aRS)-6-((((1H-tetrazol-5-yl)methyl)oxy)methyl)-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid), a GluR5 antagonist has aided in the pharmacological classification of kainate receptor-mediated effects. As such, GluR5 has been implicated in the inhibition of GABA release (Clarke *et al.* 1997). However the GluR subtype mediating the kainate-induced inhibition of glutamate release from hippocampal synaptosomes has not been elucidated (Chittajallu *et al.* 1996). Activation of these populations of kainate receptors by endogenous glutamate has not yet been verified, although recently it was shown that postsynaptic kainate receptors in CA3 pyramidal neurones can be activated during periods of repetitive stimulation (Castillo *et al.* 1997; Vignes & Collingridge, 1997). The resultant synaptic potential possesses kinetics somewhat slower than AMPA and NMDA receptor-mediated synaptic currents has recently been demonstrated.

1.4.2.4. Metabotropic Glutamate Receptors.

Metabotropic glutamate receptors (mGluRs), like mAChRs, are G-protein linked receptors and classically act via activation of PLC (group I) and inhibition of AC (group II and III) (Pin & Duvoisin, 1995) although a distinct phospholipase D (PLD)-linked mGluR has recently been described in the hippocampus (Pellegrini-Giampietro *et al.* 1996). Molecular cloning has revealed eight subtypes of mGluRs ($mGluR_{1\text{-}8}$) which are divided into three groups, based on agonist pharmacology and the signal transduction mechanisms to which they couple (Pin & Duvoisin, 1995). The major differences between the three groups are summarized in Table 1.4. 1-aminocyclopentane-1*S*,3*R*-dicarboxylic acid ((1S,3R)-ACPD) is a group I and group II mGluR agonist while L-2-amino-4-phosphonobutanoate (L-AP4) is a selective agonist at group III mGluRs (Dubner & Ruda, 1992; Thomsen *et al.* 1992; Roberts, 1995). The phenylglycine derivative (+)-α-methyl-4-carboxyphenylglycine ((+)-MCPG) is a competitive antagonist for group I and group II mGluRs (Jane *et al.* 1993; Watkins & Collingridge, 1994; Roberts, 1995; Sekiyama *et al.* 1996).

Table 1.4. mGluR subtypes: Pharmacology and transduction mechanisms.

Group	I	II	III
Subtypes	$mGluR_1$ & $mGluR_5$	$mGluR_2$ & $mGluR_3$	$mGluR_4$, $mGluR_6$, $mGluR_7$ & $mGluR_8$
Transduction Mechanisms	↑PLC (IP_3/DAG)	↓AC (cAMP)	↓AC (cAMP)
Agonists	(S)-DHPG	DCG IV	L-AP4
Antagonists	4-CPG	MCCG	MAP4

Abbreviations:

(S)-DHPG	(*S*)-3,5-dihydroxyphenylglycine
4-CPG	(*S*)-4-carboxyphenylglycine
DCG IV	(2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine
L-AP4	L-2-amino-4-phosphonobutanoic acid
MAP4	(*S*)-2-amino-2-methyl-4-phosphonobutanoic acid
MCCG	(2*S*,3*S*,4*S*)-2-methyl-2-(carboxycyclopropyl)glycine

In the hippocampus, mGluR activation, like mAChR activation has been shown to inhibit I_{AHP} and I_M (Charpak *et al.* 1990; Guérineau *et al.* 1994). In addition, mGluR activation in the hippocampus can inhibit $I_{K(LEAK)}$ which leads to an increase in input resistance that accompanies a depolarization. This mechanism is believed to be responsible for mGluR-mediated EPSPs, which are evoked following mossy fiber stimulation (Charpak & Gähwiler, 1991). It has also been demonstrated, using ACPD, that inhibition of $I_{K(LEAK)}$ in this manner can subsequently lead to the activation of a non-specific cation conductance (Guérineau *et al.* 1995). As such, many of the effects mediated by mGluRs are remarkably similar to those mediated by mAChRs. In addition, in area CA1 of the hippocampus, mGluRs inhibit the release of both GABA and glutamate (Gereau & Conn, 1995). The inhibition of glutamate release has been proposed to involve the activation of 4-AP-sensitive K^+ channels and/or via inhibition of ω-conotoxin-sensitive N-type Ca^{++} channels (Sladeczek *et al.* 1993; Swartz *et al.* 1993), although a detailed study of the mechanism of inhibition of GABA release has yet to be reported.

1.5. THE HIPPOCAMPUS

1.5.1. GENERAL

The hippocampus has proved the most popular brain region in which to study

neuronal processes, especially those related to learning and memory processes. Anatomically, this brain organ consists of an area of cortex rolled into the floor of the inferior horn of the lateral ventricle. The hippocampal formation is a highly organized structure which consists of four major regions designated Ammon's horn, dentate gyrus, entorhinal cortex and subicular complex (Fig. 1.1; Ramon y Cajal, 1891). Within each of these regions there are extensive local (Andersen *et al.* 1971; Amaral & Witter, 1989; Witter, 1989) as well as distant circuit interactions which process synaptic inputs received from extensive areas of the brain e.g. the septum and diagonal band, raphe nucleus, hypothalamus, thalamus and locus coeruleus (Swanson *et al.* 1982). Ammon's horn is itself subdivided into regions *cornu Ammon* (CA) 1–4 (Lorente de No, 1934), with the CA1 and CA3 regions forming the greater part of the hippocampus proper (Fig. 1.1). Within these regions, the cell bodies and dendrites of pyramidal neurones are organized into discrete laminae (Andersen *et al.* 1971) which can be easily distinguished in hippocampal slices prepared from transverse sections of the brain (Fig. 1.2*A*; See section 1.5.2). An extremely simplified view of the transverse connectivity between the main areas of the hippocampus is outlined by the so-called trisynaptic circuit (Andersen *et al.* 1971) which receives its major input from layers II and III of the entorhinal cortex and consists of successive activation of:

i) dentate gyrus granule cells through the lateral and medial perforant paths from the entorhinal cortex (McNaughton, 1980),

ii) CA3 pyramidal neurones via innervation from dentate granule cells through the mossy fiber pathway (Blackstad & Kjaerheim, 1961) and,

iii) CA1 pyramidal neurones via input from the axons of the CA3 pyramidal neurones through the Schaffer collateral-commissural pathway (see Fig 1.2*A*; Andersen *et al.* 1971; Swanson *et al.* 1978).

1.5.2. The Hippocampal Slice Preparation

The parallel organization of these excitatory pathways approximately transverse to the longitudinal axis of the hippocampus allows clear visibility and considerable conservation of the trisynaptic circuit following transverse slicing (Fig. 1.2*A*; Yamamoto & McIlwain, 1966; Skrede & Westgaard, 1971; Andersen, 1981).

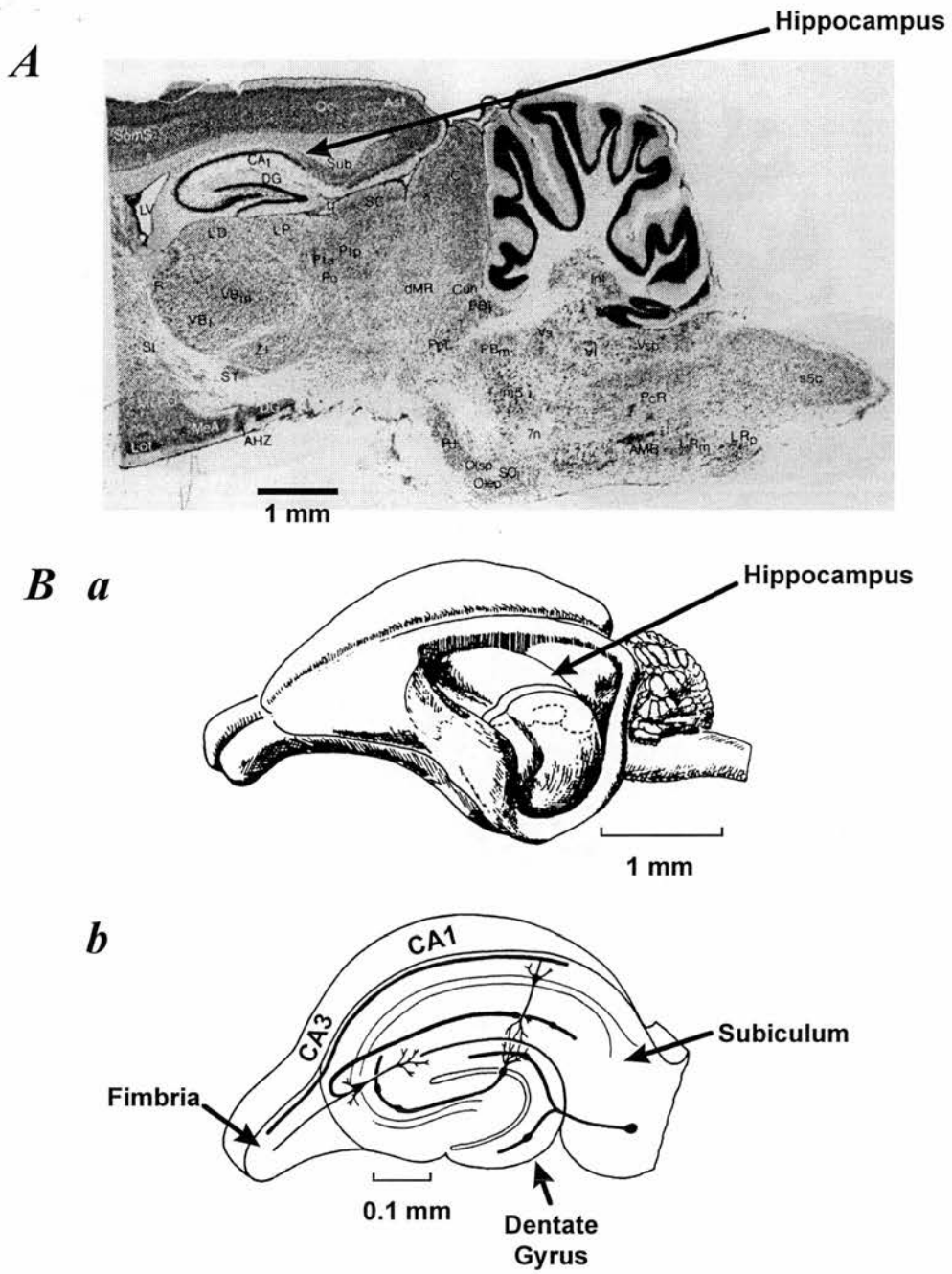

Figure 1.1. Location and General Structure of the Rat Brain and Hippocampus.

A, shows a 20 µm saggital section of rat brain in which cells are stained with Cresyl violet. The main structures within the brain are marked. CA_1: hippocampal area CA_1; DG: dentate gyrus; Sub: subiculum; LV: lateral ventricle; SomS: somatosensory cortex; Oc: occipital Area (Cortex); VB_l: lateral ventrobasal nuclear complex. *Ba*, shows a schematic representation of the rat brain and the position of the hippocampus within it. *Bb*, is a schematic representation of the transverse hippocampal slice in which the main areas (area CA1, area CA3, dentate gyrus, subiculum and fimbria) are marked. Note the different scales in each figure. Figure *A* is modified from Kruger *et al.* (1995).

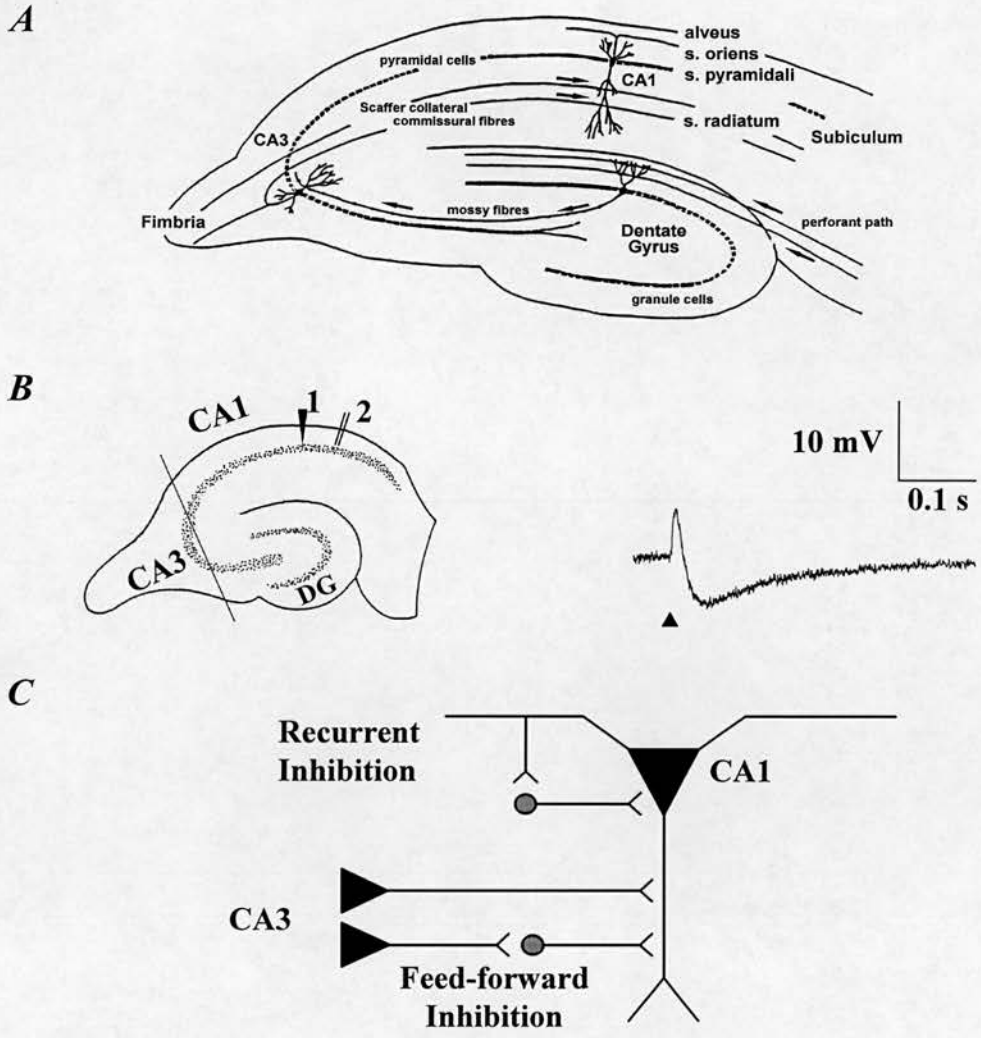

Figure 1.2. Synaptic Circuitry in the Rat Hippocampal Slice.

A, shows a schematic representation of the hippocampal slice in which the major excitatory pathways which comprise the trisynaptic circuit are marked. The direction of synaptic connectivity between pathways is represented by the arrows (perforant path → mossy fibers → Schaffer collateral-commissural fibers). Note the clear differentiation between laminae (*s. oriens*, *s. pyramidali* and *s. radiatum*). *Ba*, shows the placement of field stimulation and intracellular recording electrodes in the hippocampal slice to evoke the synaptic response shown in *Bb*. *Bb*, shows the synaptic response evoked by a single field stimulus applied to s. oriens. The point of afferent stimulation is marked by the filled triangle. Note that the stimulus evoked a depolarizing response (EPSP, mediated predominantly by AMPA receptors), followed by an hyperpolarizing response (IPSP, mediated by $GABA_A$ and $GABA_B$ receptors). Note that the NMDA receptor-mediated response is masked by the inhibitory response. *C*, shows a simplified schematic diagram of the synaptic circuitry involved in evoking the response shown in *Bb*, emphasizing the difference between recurrent and feed-forward inhibition. Pyramidal neurones are represented by filled triangles and interneurones by shaded circles. Fiber pathways are represented by solid lines and presynaptic terminals as forks. Note that all synapses are excitatory (mediated by glutamate) except those of interneurones which are inhibitory (mediated by GABA). Note that stimulation of the Schaffer collateral-commissural fiber pathway which extends from the CA3 will evoke direct excitatory responses and inhibitory responses (via inhibitory interneurones) in CA1 pyramidal neurones.

The advantages of the *in vitro* transverse hippocampal slice preparation are many-fold, including ease and stability of electrophysiological recording from identified neurones (Schwartzkroin, 1975; Schwartzkroin, 1977; Knowles & Schwartzkroin, 1981) and the ability to precisely control the temperature and composition of the extracellular medium (Andersen, 1981). Nevertheless, extrapolation to the *in vivo* situation at best may only be a close approximation due to, amongst other constraints, the substantial transection of synaptic connections between laminae as well as those to and from other brain areas. Despite this obvious limitation, the maintenance of the trisynaptic circuit and of other extrinsic inputs, in the face of gross severance of other convergent synaptic inputs, provides a preparation in which it is easy to monosynaptically activate synaptic inputs to specific target neurones (Yamamoto & McIlwain, 1966; Davies *et al.* 1990; Malinow, 1991). In this respect, the study of CA1 pyramidal neurone synaptic input has allowed the acquisition of substantial knowledge of synaptic transmission in the hippocampus.

1.6. SYNAPTIC TRANSMISSION IN THE HIPPOCAMPUS

1.6.1. SYNAPTIC TRANSMISSION IN THE SCHAFFER-COLLATERAL COMMISURAL PATHWAY

Amino acids are thought to be the principal mediators of synaptic responses evoked in CA1 pyramidal neurones following synchronous activation of Schaffer collateral-commissural afferents. During low frequency synaptic transmission (<0.1 Hz) the evoked response, like that seen at numerous other synapses in the CNS, comprises a fast EPSP followed by a biphasic IPSP (Kandel *et al.* 1961; Nicoll & Alger, 1981; Alger & Nicoll, 1982b; Fig. 1.2*B*). This fast EPSP is thought to be mediated by AMPA receptors (Davies & Collingridge, 1989; Andreasen *et al.* 1989) whilst the biphasic IPSP is mediated by $GABA_A$ ($IPSP_A$) (Schwartzkroin & Prince, 1980; Alger & Nicoll, 1982a) and $GABA_B$ ($IPSP_B$) receptors (Dutar & Nicoll, 1988c; Soltesz *et al.* 1988; Lambert *et al.* 1989), respectively. Pharmacological isolation of these responses also reveals an NMDA receptor-mediated EPSP ($EPSP_N$) which is masked predominantly by the $IPSP_A$ (Andreasen *et al.* 1989).

1.6.1.1. CA1 pyramidal neurone excitatory synaptic input

The fast excitatory input to CA1 pyramidal neurones originates from CA3 pyramidal neurones (Sayer *et al.* 1990; Malinow, 1991). Ipsilateral collaterals from these neurones synapse with CA1 pyramidal neurone apical dendrites in *s. radiatum* (Blackstad, 1956; Hjorth-Simonsen, 1973; Laurberg, 1979; Laurberg & Sorensen, 1981). These fibers form en passage excitatory synapses with CA1 pyramidal cells (Fig. 1.2*C*; Lorente de No, 1934; Andersen *et al.* 1966; Andersen & Lomo, 1966).

Under a variety of conditions, e.g. reduced synaptic inhibition (Wigstrom *et al.* 1986; Dingledine *et al.* 1986), stimulation of Schaffer collateral-commissural afferents in *s. radiatum* activates both $EPSP_N$s and $EPSP_A$s. It is likely, therefore, that the release of a single neurotransmitter (L-glutamate) from Schaffer collateral-commissural terminals accounts for both these synaptic potentials.

1.6.1.2. CA1 pyramidal neurone inhibitory synaptic input.

Historically, it was believed that inhibitory synaptic input to CA1 pyramidal neurones was mediated solely through classical recurrent (feedback) inhibitory circuits (Fig. 1.2*C*; Kandel *et al.* 1961; Andersen *et al.* 1964; Dingledine & Langmoen, 1980; Knowles & Schwartzkroin, 1981). Subsequently, it was realized that there is an additional feedforward inhibitory circuit that contributes to synaptic inhibition (Fig. 1.2*C*; Alger & Nicoll, 1982b; Schwartzkroin & Knowles, 1983; Buzski, 1984). In both types of inhibition, AMPA and NMDA receptor activation provide the major synaptic drive of the inhibitory interneurones that mediate the final GABAergic input to the CA1 pyramidal neurones (Davies & Collingridge, 1989; Andreasen *et al.* 1989; Sah *et al.* 1990).

Both recurrent and feedforward GABAergic interneurones evoke a classic fast hyperpolarizing $IPSP_A$. This was initially characterized by its sensitivity to bicuculline and to both intra- and extra-cellular Cl^- ion concentrations (Kandel *et al.* 1961; Andersen *et al.* 1964; Allen *et al.* 1977; Eccles *et al.* 1977; Dingledine & Langmoen, 1980; Dingledine & Gjerstad, 1980).

The notion that GABA was the neurotransmitter mediating this IPSP was confirmed

by:

i) the similarity between the properties of this IPSP and those of the hyperpolarization induced by iontophoretic application of GABA to the soma of CA1 pyramidal neurones (Curtis *et al.* 1970b; Andersen *et al.* 1980; Alger & Nicoll, 1982a),

ii) the presynaptic location of GABA in hippocampal interneurones (Anderson *et al.* 1986; Gamrani *et al.* 1986; Woodson *et al.* 1989), and

iii) the Ca^{++}-dependent release of GABA from the hippocampal CA1 region (Skrede & Malthe-Sorenssen, 1981; Spencer *et al.* 1981; Corradetti *et al.* 1983).

Feedforward inhibitory circuits additionally activate a $GABA_B$ receptor-mediated slow $IPSP_B$ (Nicoll & Alger, 1981; Newberry & Nicoll, 1984a), which was originally characterized by its similarity to (-)-baclofen-induced hyperpolarizations (i.e. both exhibited similar I-V relationships and dependency on extracellular K^+) (Newberry & Nicoll, 1984b; Newberry & Nicoll, 1985). This was subsequently confirmed by the inhibition of $IPSP_B$s by selective $GABA_B$ receptor antagonists (Lambert *et al.* 1989; Olpe *et al.* 1990).

1.6.2. The Septohippocampal Input

As well as such intrinsic synaptic inputs, CA1 pyramidal neurones in the hippocampus receive a number of extrinsic synaptic inputs from diverse areas of the brain. One of the best-characterized extrinsic synaptic inputs is the septohippocampal input (Dutar *et al.* 1995). This comprises a heterogeneous population of afferents that mediate their effects through the release of numerous neurotransmitters including ACh, GABA and a variety of neuropeptides (Decker & McGaugh, 1991; Dutar *et al.* 1995). Cholinergic neurones in the medial septum and nucleus of the diagonal band of Broca are the main groups of neurones to innervate the hippocampal formation (Fig. 1.3). Of the heterogeneous inputs, the cholinergic aspect of the septohippocampal input to the hippocampus has probably received most attention due to its involvement in mnemonic processing (Cole & Nicoll, 1983;

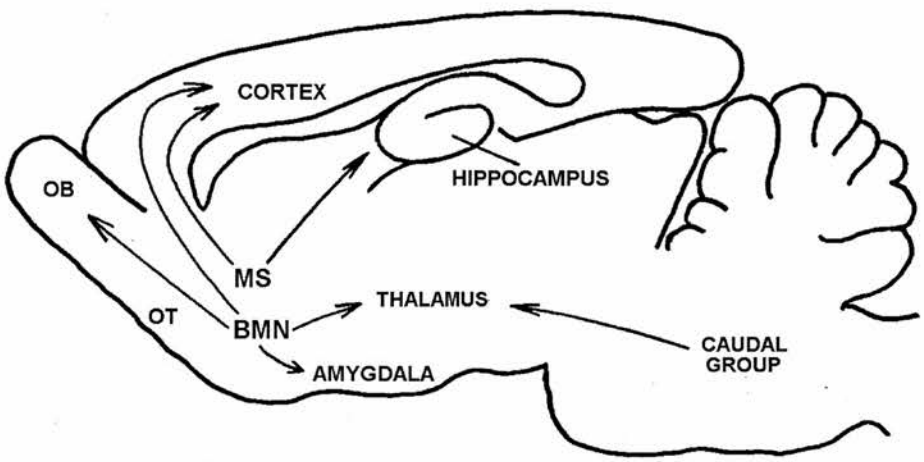

Figure 1.3. The Main Cholinergic Pathways in the Rat Brain.

This figure shows a schematic representation of the rat brain in saggital section. The main cholinergic projections represented by the solid arrows. **OB**: olfactory bulb; **OT**: olfactory tubercle; **MS**: medial septum; **BMN**: basal magnocellular nuclei. Note the septohippocampal cholinergic pathway runs between the medial septum and the hippocampus.

Decker & McGaugh, 1991; Dutar *et al.* 1995). In this respect, this pathway and the cholinergic system are thought to be involved in the control and generation of the hippocampal θ-rhythm (see section 1.1.4.3). Stimulation of this input has been shown to activate a slow EPSP and to reduce SFA via mAChR-mediated inhibition of K^+ conductances (Cole & Nicoll, 1983; Cole & Nicoll, 1984a; Madison *et al.* 1987; Segal, 1988; Pitler & Alger, 1990).

1.7. AIMS

The initial aim of this project was to establish an experimental protocol with which to evoke isolated cholinergic synaptic responses in CA1 pyramidal neurones of the rat hippocampus. This was to be achieved using the established method of stimulation of the *s. oriens* to evoke the release of ACh from septohippocampal cholinergic afferents in area CA1 (Cole & Nicoll, 1983 & 1984). Ionotropic glutamate and GABA receptor antagonists were to be used to block all "fast" amino acid-mediated synaptic transmission. Once isolated the intention was to develop a protocol to evoke consistent cholinergic synaptic responses with the simplest of stimulation paradigms. Subsequently, a basic characterization of cholinergic synaptic responses would be required to establish the cholinergic nature of the responses. Having achieved this, experiments would be conducted to investigate possible mechanisms for the modulation of this system.

A common mechanism of control of synaptic inputs within the brain is via activation of different receptors to those that mediate the postsynaptic response, e.g. activation of autoreceptors, or presynaptic heteroreceptors (Thompson *et al.* 1993). The primary aim of this study, therefore, was to investigate the possibility that a neurotransmitter/neuromodulator other than ACh may restrict the activation of the mAChR-mediated slow EPSP. Initially adenosine was chosen, because this classic neuromodulator plays an important role in controlling the excitability of neuronal networks by inhibiting other excitatory synaptic inputs in the hippocampus, e.g. glutamate (Thompson *et al.* 1992; Thompson *et al.* 1993). Modulation of cholinergic responses by other neurotransmitters was also considered.

CHAPTER 2

GENERAL METHODS

2.1. EXPERIMENTAL PREPARATION

2.1.1. GENERAL

All experiments were performed using standard intra- and extra-cellular electrophysiological techniques. Synaptic recordings were made from the CA1 region of transverse coronal rat hippocampal slices obtained from 2–5 week old female Cobb-Wistar rats.

2.1.2. PREPARATION OF HIPPOCAMPAL SLICES

The rats were cervically dislocated and subsequently decapitated in accordance with UK Home Office guidelines. The brain was removed rapidly and placed immediately in ice cold (0–4°C) artificial cerebrospinal fluid (ACSF). The brain minus the cerebellum was hemisected and an agar block fixed to the base of the brain using superglue. The brain was then fixed to a polypropylene block for the subsequent cutting of 400 μm thick transverse coronal slices using a vibroslicer (Campden Instruments, Loughborough, UK). Throughout the slicing procedure the brain and slices were held in a chamber containing ice cold, ACSF. The slices were then transferred to a glass Petri dish containing ACSF at room temperature (18–24 °C). The hippocampus was cut away from the rest of the brain slice and the CA3 region cut away to eliminate changes in network function that can occur due to epileptiform bursting in area CA3 when picrotoxin is applied to the slice (see Fig. 3.1). Following an incubation period of at least half an hour the resultant CA3-ectomized slices were placed in the recording chamber on a nylon mesh at the interface of a warmed (32 ± 2 °C), perfusing (1–3 ml min^{-1}) ACSF and an oxygen-enriched (95% 0_2, 5% CO_2; BOC Medical Gasses, UK), humidified atmosphere (Fig. 2.1). The slices were then allowed to equilibrate in this environment for approximately 1–2 h before any electrophysiological recording was attempted. Surplus slices contained in the Petri dishes remained viable for several hours. If required, these slices could be transferred to the recording chamber at a later time.

The standard perfusion medium comprised (in mM): NaCl, 124; KCl, 3; $NaHCO_3$, 26; NaH_2PO_4, 1.25; $CaCl_2$, 2; $MgSO_4$, 1; D-glucose, 10; and was bubbled with 95% 0_2, 5% CO_2 to maintain a pH of 7.4–7.5. ACSF was made up using distilled water filtered (10–18 MΩ Cm^{-1}) using the Millipore Milli-Q filter system (Millipore; Molsheim, France) and all chemicals were analaR grade (BDH Chemicals Ltd., Poole, UK).

2.2. RECORDING SET-UP

The recording chamber and the micromanipulators were mounted on magnetic stands (Narishige, Japan) supported on a steel plate on an anti-vibration table (Ealing, MA, USA). A steel Faraday cage, grounded through the amplifier (Axoclamp-2B, Axon Instruments, CA, USA), was placed around the recording system in order to isolate it from extraneous electrical noise. All electrical equipment that was fitted with earth leads was secured in a rack and earthed through the mains.

The recording chamber was a modified version of an interface electrophysiological recording chamber designed by (Spencer *et al.* 1976) (Fig. 2.1). It consisted of an outer water bath with a centrally mounted platform on which the tri-compartmental recording chamber was secured. The outer water bath was partially filled with distilled water and was bubbled with a 95% O_2, 5% CO_2 mixture. The height of the covering lid was adjusted to allow the oxygen-enriched humidified atmosphere to flow over the slices.

ACSF was pumped, by means of a peristaltic pump (Watson-Marlow, England), to a gravity-feed syringe. This fed a constant flow (1–3 ml min^{-1}) of ACSF into the first well of the recording chamber. The ACSF then flowed around the circumference of this well and through a small inlet into the recording chamber proper. Here, it flowed underneath the nylon mesh and was removed through a separate outlet by suction through a syringe needle connected to a water-powered suction pump (Brownall, England). By adjusting the height of the gravity feed and the magnitude of the outlet suction, the level of ACSF in the recording chamber was maintained so that it was in contact with the mesh but did not cover the slices. A heated patch connected to a constant current source (Maplin XG89, Taiwan) was attached to a metal plate in the

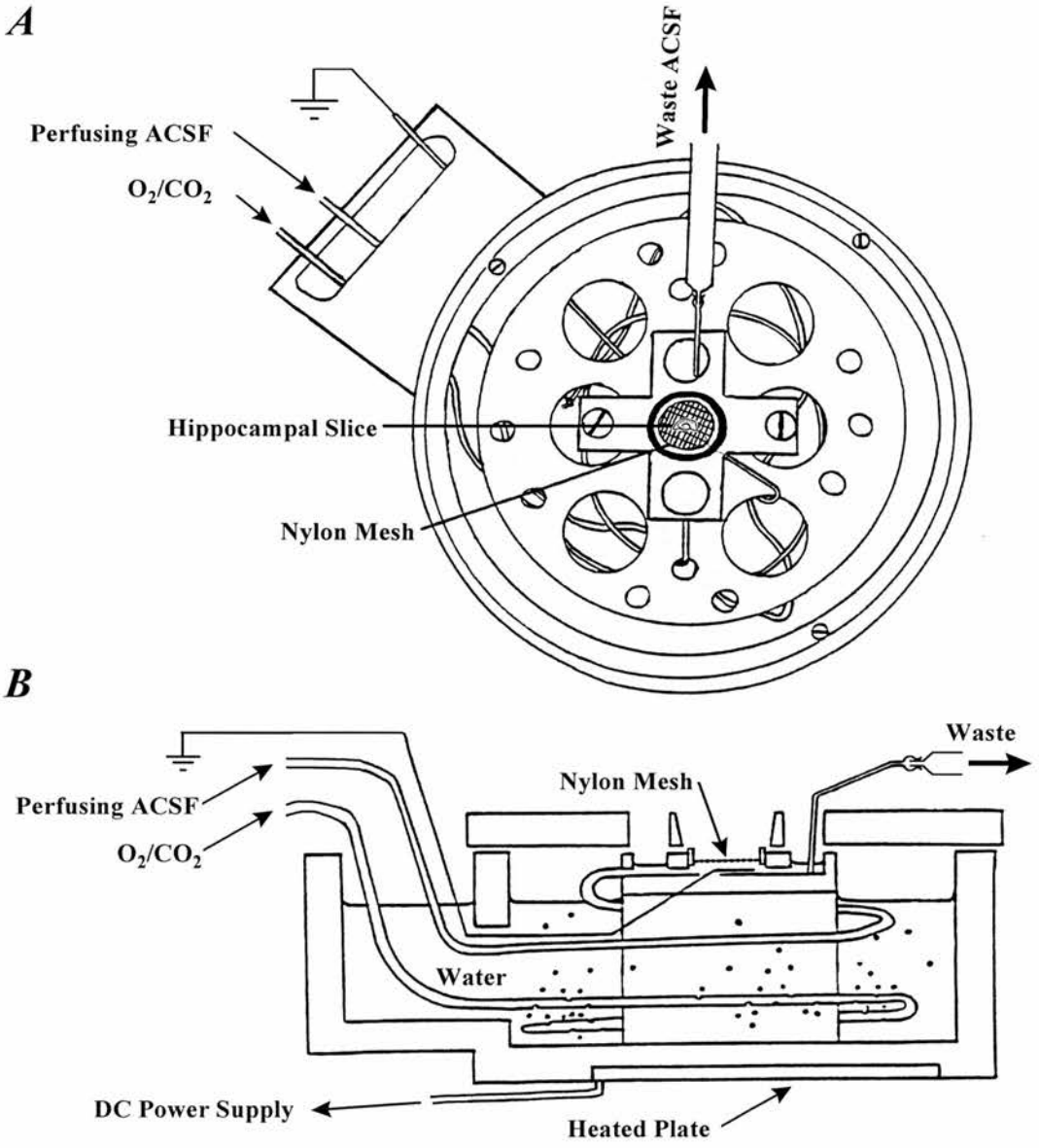

Figure 2.1. The interface recording chamber.

This figure shows a schematic representation of the recording chamber as viewed from above (*A*) and from the side (*B*). ACSF, perfused with 95 % O_2/ 5 % CO_2 and maintained at 32 °C, was passed through tubing immersed in the temperature controlled external water bath and into the interface chamber. The level of ACSF in the interface chamber was maintained just above the level of the nylon mesh which supported the hippocampal slice. Waste ACSF was drawn off by suction through a hypodermic needle. The humidified atmosphere was maintained by passing 95 % O_2/ 5 % CO_2 though distilled water maintained at 32 °C (by a heated plate) to create a fine mist. The reference electrode was grounded through the Axoclamp 2B.

base of the water bath (Fig 2.1) to maintain the temperature at 32 ± 2 °C.
The slices in the recording chamber were epi-illuminated with a fiber optics system (Nachet, France). All stimulating and recording electrodes were mounted on micromanipulators (Narishige, Japan) which allowed movement in the *x*, *y* and *z* axes. An additional single axis fine movement hydraulic manipulator (Narishige, Japan) was used for the recording electrode to allow increased sensitivity when advancing the electrode for the impalement of neurones. To enable the positioning of electrodes in the slice, the slice was viewed using an overhead dissecting microscope (M3C, Leica, England). Recording microelectrodes were placed, at an angle of approximately 45° to the vertical, in either *stratum oriens* (CA1 pyramidal basal-dendritic layer) or *stratum radiatum* (CA1 pyramidal cell apical dendritic layer).

2.3. RECORDING TECHNIQUES

2.3.1. RECORDING ELECTRODES

Microelectrodes were pulled from thick walled (internal diameter: 0.69 mm; outer diameter: 1.2 mm) borosillicate glass capillaries with an inner filament (120F-10, Clark Electromedical Instruments, England), on a horizontal Flaming-Brown P-97 micropipette puller (Sutter Instruments Co., USA). Intracellular microelectrodes were back-filled with 2 M potassium methylsulphate (ICN Biomedicals Inc., USA) and had resistances ranging 60–120 MΩ. Extracellular microelectrodes with resistances ranging 1–5 MΩ were back-filled with 4 M sodium chloride.

Recording microelectrodes were then mounted in electrode holders (Clark Electrochemical Instruments, England) filled with 4 M sodium chloride, in contact with a silver chloride coated silver wire. The holders were inserted into unity gain head stages (current gain x 0.1 or x 1.0: Axon Instruments, CA, USA) and connected to an Axoclamp-2B amplifier through the microelectrode 1 (ME1) port for use in "bridge balance" or "discontinuous current clamp" (DCC) modes. A silver-silver chloride bath reference electrode, submerged in the recording chamber, was also connected to the head stage and grounded through the Axoclamp amplifier.

2.3.2. INTRACELLULAR RECORDING

Impalement of CA1 pyramidal neurones was achieved by manually advancing the recording microelectrode through the slice using the hydraulic vertical axis manipulator, and intermittently applying a 1–2 ms "buzz" on the Axoclamp-2B. "Buzzing" momentarily increases the capacitance neutralization of the electrode and causes the headstage to oscillate at high frequency. This procedure aids penetration of cells when the electrode is opposed to the cell membrane although the mechanism by which this occurs is unclear. Cell impalement was routinely performed in normal ACSF and only after a stable recording was obtained for at least 10 min were drugs administered.

The membrane potential (V_m) of the neurone (as measured by the Axoclamp 2B amplifier) was measured as the potential difference between the microelectrode and the bath reference electrode. Prior to searching for cells, when the microelectrode had been positioned extracellularly in the slice, V_m was set to zero. Immediately following the end of each experiment this reading was checked again and any correction in the recorded membrane potential made.

2.3.3. CURRENT INJECTION

Before searching for cells the resistance of the microelectrode was measured in bridge balance mode. This was done by balancing out the unwanted potential drop across the microelectrode resistance through a differential amplifier incorporated in the Axoclamp 2B amplifier (Fig. 2.2). The mode of recording was then switched to discontinuous current clamp mode which allowed rapid switching (frequency 3–5 kHz) between current injection and voltage recording. Thus, current was injected during the first 30 % of the cycle and the resulting potential across the microelectrode resistance due to charging continually monitored on an oscilloscope (Fig. 2.2). The capacitance neutralization was increased so that when the cell V_m was sampled, just prior to the next current injection, the potential across the microelectrode tip had fully decayed (Fig 2.2). Low resistance microelectrodes (60–90 MΩ) allowed the most accurate recording of membrane potential during current injection.

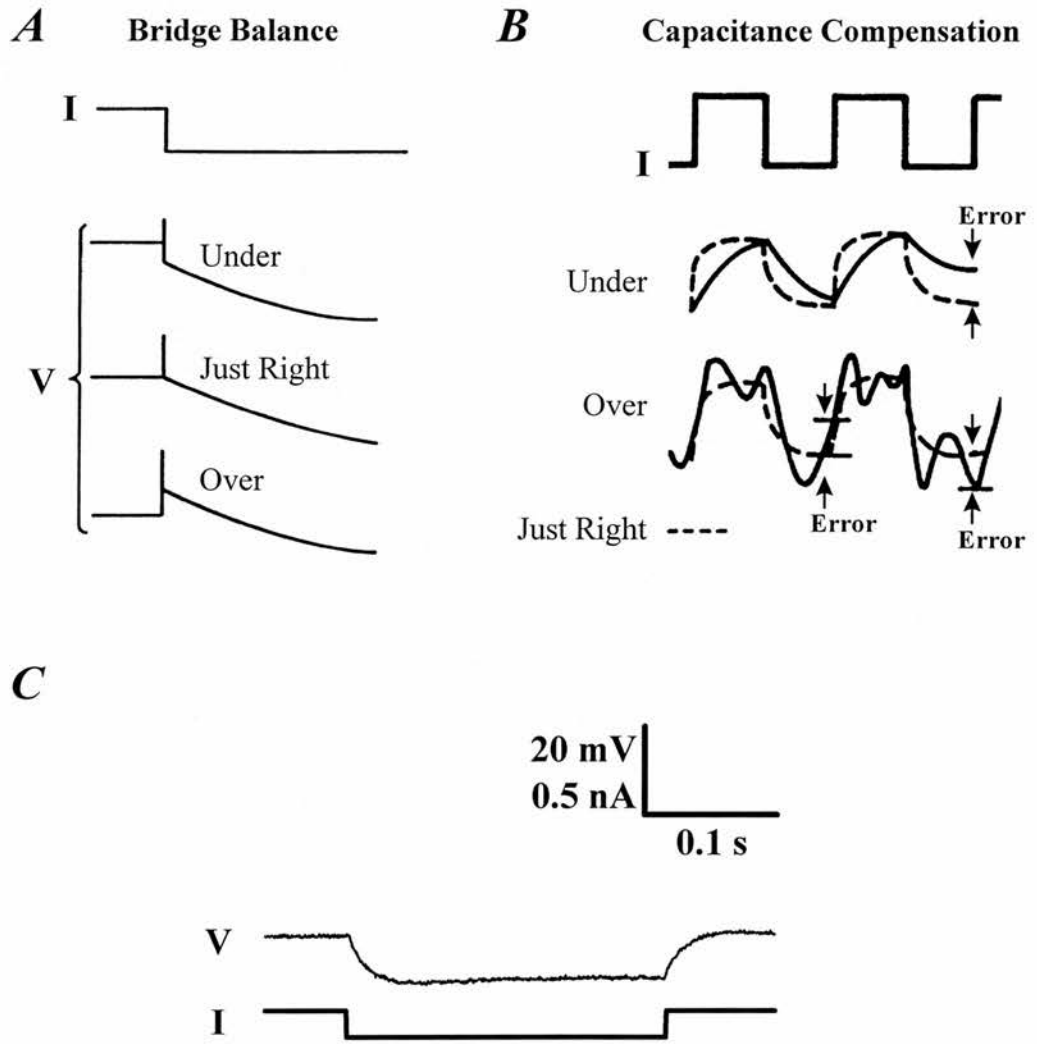

Figure 2.2. Bridge balance and DCC recording.

A, is a schematic diagram of the voltage responses (V) to hyperpolarizing current steps (I) with varying degrees of bridge balance applied. Note that when the resistance of the electrode is properly balanced ("just right") there is no voltage error at the start of the current step but a capacitance transient can be seen at this point. *B*, shows plots of the potentials (V) which develop across the resistance of the electrode during injection of depolarizing current pulses (I) (switching frequency 3 kHz). Current is injected during the first part of the cycle and membrane potential is "sampled" at the points indicated by the arrows. The dashed line represents the ideal capacitance compensation ("just right"), while solid lines represent the voltage change when capacitance is over- or under-compensated. Note that during current injection the electrode capacitance "charges" and then "discharges". The membrane potential is sampled just before the next current injection. As such, errors (indicated by the arrows) can be introduced if the capacitance is under- or over-compensated (indicated by the solid lines). *C*, shows an intracellular voltage (V) response from a CA1 pyramidal neurone in response to a hyperpolarizing (negative) current step recorded in discontinuous current clamp (DCC) mode. Note that the electrode capacitance has been completely compensated, such that no capacitance transient is observed. Figures *A* and *B* are adapted from Halliwell & Whittaker (1987).

2.3.4. AMPLIFICATION AND FILTERING

Synaptic potentials recorded through the ME1 port on the Axoclamp-2B amplifier were amplified 10 fold by an in-built gain. In DCC mode the amplifier set the sample rate (3–5 kHz switching frequency). Signals above 1–3 kHz were filtered, using a low-pass filter, which did not noticeably affect the waveform of the synaptic potentials. Secondary amplification of synaptic responses was provided by variable gain DC amplifiers (Neurolog, Digitimer, England). The output signals were then digitally filtered through a Digidata 1200 interface (Axon Instruments Ltd.) connected to a Dell Dimension P75t IBM personal computer (PC) (Dell, Texas, USA) (Fig. 2.3). Any further filtering was carried out during off-line analysis with Clampfit software (Axon Instruments Ltd.).

2.3.5. DATA DISPLAY AND STORAGE

Digitized data were captured and simultaneously viewed using pClamp6 software (Axon Instruments Ltd.) on the PC. Digitized records were stored on the hard disk of the PC for off-line analysis using Clampfit software (Axon Instruments Ltd.). The potential across the microelectrode in discontinuous current clamp mode was monitored on an analogue oscilloscope (Phillips, Holland). A continuous chart record or digital tape (DAT) record of the membrane potential of the cell was captured by a chart recorder (Gould, Ilford, Essex, UK) or DAT recorder (DTR1404, Biologic Scientific Instruments, Claix, France). This provided a means to assess the passive stability of the cell throughout each experiment.

2.3.6. EXTRACELLULAR RECORDING

Extracellular field potential recordings from the apical dendritic layer were made with respect to the reference electrode (Fig. 2.4). Data were displayed and stored in a similar manner to that used for intracellular recording. In experiments investigating the effect of drugs on the slope of the field EPSP, the test field EPSP size was adjusted to approximately 50 % of maximum.

2.4. STIMULATION

The septo-hippocampal cholinergic input or the Schaffer collateral-commissural fiber

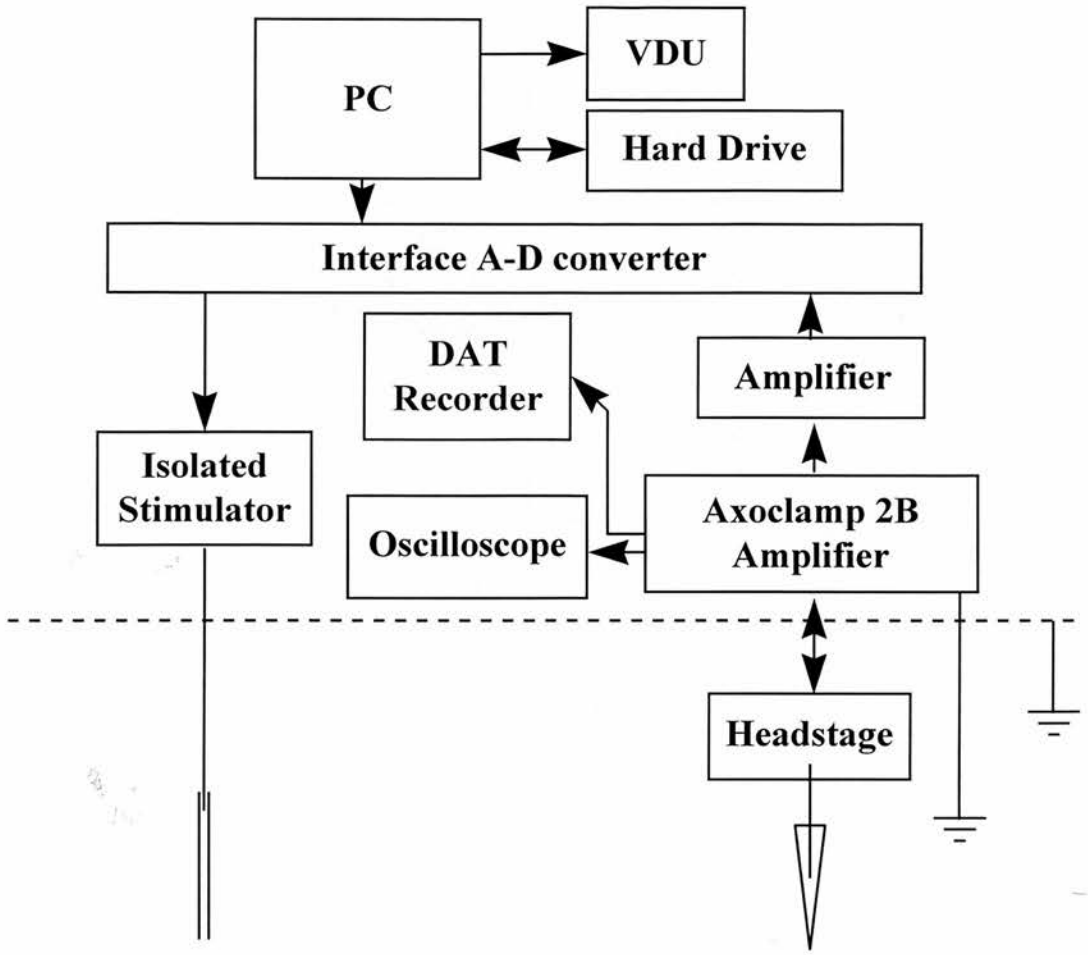

Figure 2.3. The recording set-up.

This figure shows a flow diagram of the experimental apparatus used for electrophysiological recording. The direction of information transfer is represented by the direction of the arrows. The dashed line represents the Faraday cage which surrounds the recording chamber and screens the recording system from extraneous electrical noise. The stimulating electrode is represented by the parallel lines attached to the stimulator and the recording electrode is represented by the open triangle connected to the headstage. Data were displayed on the oscilloscope and VDU and stored on DAT tape and on the hard drive of the PC. Abbreviations are, A-D converter: analogue to digital converter; DAT recorder: digital data recorder; PC: personal computer; VDU: visual display unit.

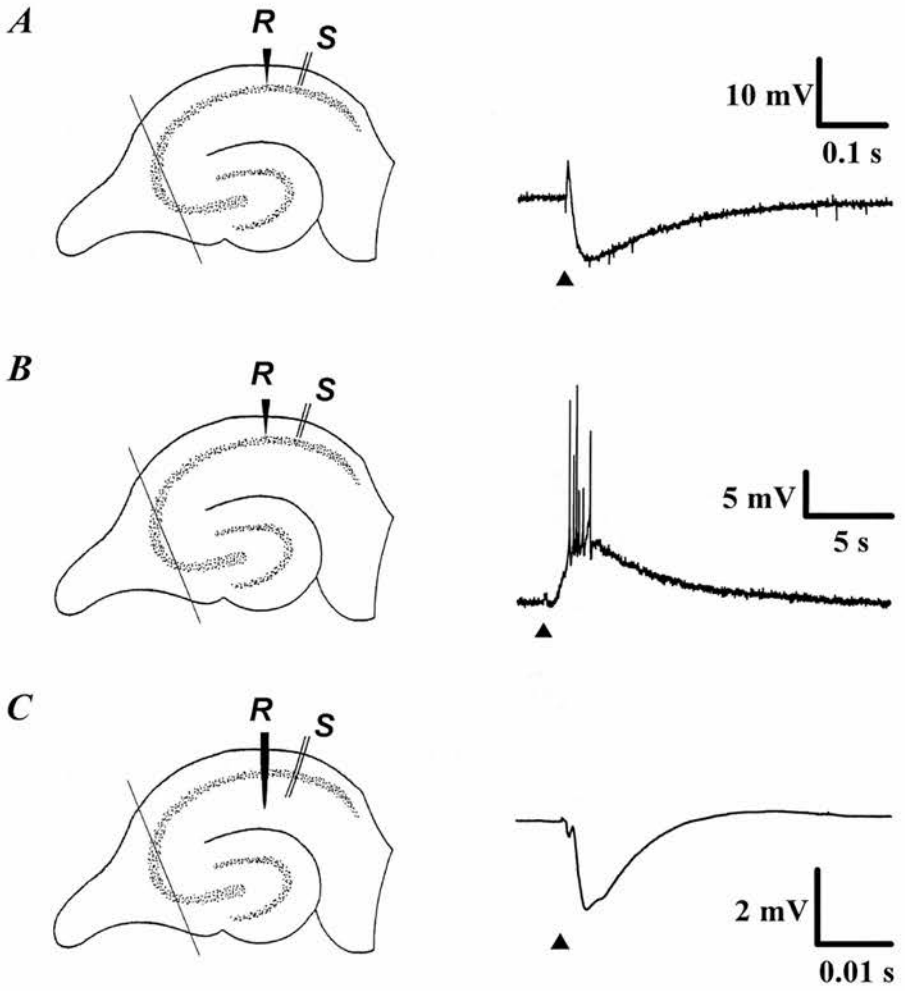

Figure 2.4. Electrode positioning for generation of synaptic responses in the rat hippocampal slice.

Figures *A*-*C*, show the placement of stimulating (*S*) and recording (*R*) electrodes in the hippocampal slice (left hand panels) and the responses obtained using intracellular (*A* and *B*) and extracellular recording (*C*) (right hand panels). *A*, shows the placement of a stimulating electrode in *s. oriens* (*S*) to evoke a fast EPSP followed by an IPSP (mediated by glutamate and GABA respectively) measured using an intracellular recording electrode (*R*) from a CA1 pyramidal neurone (V_m = -64 mV; see section 1.6.1). A similar response can be obtained on stimulation of Schaffer collateral-commissural fibers in *s. radiatum* (see Fig. 3.1). *B*, also shows placement of a electrodes in the same configuration as in *A*, to evoke a slow mAChR-mediated EPSP ($EPSP_M$; see chapter 3). *C*, shows placement of a stimulating electrode (*S*) in *s. radiatum* to evoke a field EPSP which is mediated by glutamate, recorded using an extracellular field electrode (*R*) placed in the dendritic field *s. radiatum*.

input to CA1 pyramidal neurones were orthodromically stimulated using bipolar stimulating electrode(s) which were placed on the surface of the slice in *stratum radiatum* and *oriens* respectively. The stimulating electrodes consisted of two 50 μm diameter Formvar insulated nickel-chromium (80 %: 20 %) wires (Advent Research Materials Ltd., England) twisted together and cut at the end to provide a focal stimulation. Stimuli were produced by constant voltage or constant current isolated stimulator boxes (Digitimer, England) in turn stimulated by a 5 V pulse produced by the PC and relayed via the Digidata 1200. In every series of experiments stimuli comprised square-wave pulses (20–200 μs; 0.1–30 V or 0.1–10 mA) delivered homosynaptically at a fixed intensity every 15 s for ionotropic glutamate and GABA receptor-mediated responses and every 5–10 min for mAChR-mediated responses.

2.5. SLICE AND CELL SELECTION CRITERIA

For both intra- and extra-cellular recordings slices were chosen that evoked only a single population spike to a low frequency stimulus (0.033 Hz) in standard ACSF. This was taken as an indicator for healthy synaptic inhibition. For intracellular recordings the input resistance of the cell was greater than 30 MΩ measured as described in section 2.6 below. Action potentials overshot 0 mV and the cells exhibited some degree of SFA (an accommodation of action potential firing when a positive current step e.g. + 0.3-0.5 nA, 300ms was applied to the cell). In all experiments care was taken that both the recorded response and the passive membrane properties of intracellularly recorded neurones were stable for a period of 10–15 min before any physiological or pharmacological protocol was employed.

2.6. EXPERIMENTAL DESIGN

During the period between stimuli the input resistance and the extent of SFA of each neurone were measured routinely using 300–600 ms long negative and positive current steps (± 0.1–0.4 nA), respectively. In all experiments in which $EPSP_M$s (Fig. 2.4), or sub-threshold stimulation induced reductions in SFA, were evoked, baseline recordings comprised either successive EPSPs which had peak amplitudes that differed by no more than 15 %, or reductions in SFA, that were consistent over 20–30 min, respectively. In all experiments where the effects of drugs on V_m were

assessed, stable baseline recordings, where the V_m and input resistance of the cell varied no more than 1 mV and 10 % respectively over a period of 10–30 min, were obtained prior to application of the drug.

In other experiments where it was required to compare the EPSPs evoked in the presence and absence of a drug at the same V_m, DC was injected through the electrode to compensate for any drug-induced changes in membrane potential.

2.7. ANALYSIS OF DATA

Analysis of intracellularly recorded responses usually comprised the measurement of the peak amplitude or the slope of the rising phase of the recorded response (extracellular field-EPSPs). The synaptic responses analyzed were averages of four successive responses in the case of ionotropic glutamate and GABA receptor-mediated responses or single responses in the case of mAChR-mediated responses. Average responses were used to reduce noise and mean the effects of biological variation.

The data-handling software package Sigmaplot (Version 2.01; Jandel Scientific, USA) was run on an IBM PC (Dell) and was used to generate single plots and plots of means. Pooled data are presented as means ± standard error of the mean (S.E.M.) and statistical significance was assessed using a paired or unpaired Students *t*-test performed on raw data with $P < 0.05$ being taken as indicating statistical significance. *n* values refer to the number of times a particular experiment was performed, each in a different slice taken from a different rat.

Where concentration-response data has been illustrated and curve fitting used, data (Y) were fitted to the logistic expression: $Y = M\,(X^P/[X^P + K^P])$; where X is the concentration of CADO, M the maximum effect, K is the IC_{50} or EC_{50} value and the power P determines the slope of the curve.

2.8. BIOCYTIN STAINING

When depolarising current pulses were employed, intracellular diffusion of biocytin from the electrode resulted in adequate filling of the recorded neurones. Following recording, slices were sandwiched between two Millipore filters and fixed in 2.5 %

paraformaldehyde, 1.25 % glutaraldehyde and 15 % (v/v) picric acid in 0.1 M phosphate buffer (PB; pH 7.4), usually overnight. After fixation, the tissue was rinsed in phosphate buffer and infiltrated with 10 % and then 20 % sucrose solution. To increase the penetration of the reagents, slices were snap-frozen in liquid nitrogen and thawed in phosphate buffer. Slices were then embedded in 10 % gelatine which was placed in the same fixation solution for approximately one hour or until the block was sufficiently hard for cutting. Taking care to keep the block flat, slices were re-sectioned on a vibratome at 50–60 μm thickness. After three 10 min washes in phosphate buffer and two 10 min washes in Tris-buffered saline (TBS; 0.05 M, pH 7.4), the sections were incubated overnight at 4 °C with 1 % avidin-biotinylated horseradish preoxidase complex (ABC, Vector laboratories). Excess ABC was removed by three 20 min washes in TBS and two 20 min washes in Tris-buffer (pH 7.6). Sections were then developed for 10–15 min in Tris-buffer with 0.05 % 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.01 % hydrogen peroxide. After excess DAB was removed with further washes in tris-buffer (1 x 15 minutes) and phosphate buffer (2 x 10 minutes), sections were postfixed for 1 hour with 1 % osmium tetroxide in phosphate buffer. Following one 10 min rinse in phosphate buffer followed by three 10 min washes in distilled water, slices were block-stained for 40 min in a filtered 1 % aqueous solution of uranyl acetate. Sections were then washed in distilled water (2 x 10 min) before being dehydrated between coverslips in an ascending series of ethanol solutions (50 %, 70 %, 95 %, 100 % x 2; 10 minutes in each). From the second absolute alcohol step, sections were transferred to propylene oxide (2 x 10-minutes). Material was then infiltrated overnight in Durcupan resin (Fluka) before being mounted onto glass slides and transferred to an oven (56 °C, 48 h) to allow the resin to resin polymerise.

2.9. DRUGS

Drugs were stored frozen in stock aliquots (100 μl to 5 ml) of 100 to 10000 times final concentration and dissolved in ACSF, deionised water, 25 mM NaOH or ethanol. All drugs were added to the control ACSF and administered by bath perfusion for at least 15 min to allow their full equilibration within the slice.

Adenosine, atropine, baclofen, diphenylmethanone, 8-bromoadenosine 3,5-cyclic monophosphate (8-Br cAMP), 2-chloroadenosine (CADO), carbachol (CCh), 2-chloro-N^6-cyclopentyladenosine (CCPA), nitrendipine, physostigmine, picrotoxin and $R(-)N^6$-(2-phenylisopropyl) adenosine (R-PIA) were purchased from Sigma (Poole, UK). 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX), dipyridamole (DPY), 6-nitro-7-sulphamoylbenzo[*f*]quinoxaline-2,3-dione (NBQX) were purchased from Tocris Cookson Ltd. (Bristol, UK). D-2-Amino-5-phosphonopentanoate (AP5), 2-*p*-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine (CGS 21680), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 5-iodotubercidin (5-IT), (+)-α-methyl-4-carboxyphenylglycine ((+)-MCPG) and nitrendipine were purchased from Research Biochemicals International (MA, USA).

D-(*E*)-2-Amino-4-methyl-5-phosphono-3-pentanoic acid (CGP 40116), *p*-[3-aminopropyl]-*p*-diethoxymethylphosphinic acid (CGP 35348) and [1-(*S*)-3,4-dichlorophenyl)ethyl]amino-2-(*S*)-hydroxypropyl-p-benzyl-phosphonic acid (CGP 55845A) were gifts from Ciba-Geigy Ltd. (now Novartis; Basle, Switzerland). Diphenylmethanone, 0-[2-(3-carboxy-1,2,5,6-tetrahydro-1-pyridinyl)ethyl]oxime hydrochloride (NNC 05-0711) was a gift from Novo Nordisk, Denmark. 11-2[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11,-dihydro-6H-pyrido[2,3b] [1,4]benzodiazepin-6-one (AFDX 116) was a gift from Boehringer Ingelheim (Berkshire, UK).

CHAPTER 3

ACTIVATION OF MUSCARINIC RECEPTOR-MEDIATED SYNAPTIC RESPONSES IN THE RAT HIPPOCAMPUS *IN VITRO*

3.1. INTRODUCTION

As reported in the chapter 1, CA1 pyramidal neurones in the hippocampus receive an extrinsic synaptic input from the medial septum known as the septohippocampal input (Dutar *et al.* 1995). High frequency stimulation of this input, in addition to activating multiple glutamatergic and GABAergic synaptic responses, has been shown to activate a slow excitatory postsynaptic potential (slow EPSP) and to reduce SFA via mAChR-mediated inhibition of $I_{K(LEAK)}$ and I_{AHP} respectively (Cole & Nicoll, 1983; Cole & Nicoll, 1984a; Madison *et al.* 1987; Segal, 1988; Pitler & Alger, 1990).

The prolonged excitation produced by synaptic activation of mAChRs on CA1 pyramidal neurones, if left unregulated, could be detrimental to these cells and may result in epileptogenesis (Lothman *et al.* 1991; Wasterlain *et al.* 1993). In addition, the ability of ACh to modulate postsynaptic action potential firing properties may underlie cholinergic effects on learning and memory function (Cole & Nicoll, 1984a; Figenschou *et al.* 1996). As such, understanding of such responses may be of some importance with regard to diseases where memory and cholinergic systems are affected such as Alzheimer's disease (Giacobini, 1990; Fibiger, 1991; Thal, 1996).

The initial aim of this chapter was to extend the scope of previous studies by developing a simple method with which to evoke reproducible cholinergic synaptic responses which were pharmacologically isolated from other "fast" excitatory and inhibitory synaptic responses. In addition, this chapter presents a pharmacological profile of synaptic responses evoked by stimulation in *s. oriens*. Where possible data is presented to demonstrate preliminary evidence for previously unreported responses to synaptic activation of cholinergic afferents.

3.2. RESULTS

3.2.1. General Properties of CA1 Pyramidal Neurones

Recordings in this and subsequent chapters were made from CA1 pyramidal neurones (Fig. 3.1*B*) in the rat hippocampus and, unless otherwise stated, were made using the intracellular recording techniques described in chapter 2. These neurones had a mean recorded input resistance of 50.8 ± 0.2 MΩ and a mean resting V_m of -59 ± 1 mV ($n = 29$). Typical cellular responses to positive and negative current steps evoked in these neurones are illustrated in figure 3.1 *C*.

3.2.2. Amino Acid Receptor-Mediated Responses

Single shock stimulation applied in *stratum radiatum* or *stratum oriens* (Fig. 3.1*A*) evoked an EPSP that was followed by an IPSP (Fig. 3.1*D*). It was possible to isolate each component of the EPSP and IPSP using different combinations of AMPA, NMDA, $GABA_A$ and $GABA_B$-receptor antagonists (Fig. 3.2*B-E*). As illustrated in Table 3.1 and Figure 3.2 the response was made up of a fast $EPSP_A$ (Fig. 3.2*B*) which was followed by a slower $EPSP_N$ (Fig. 3.2*C*) which was normally masked by a fast $IPSP_A$ (Fig. 3.2*D*) and a slower $IPSP_B$ (Fig. 3.2*E*). The AMPA and NMDA receptor-mediated responses were inhibited by NBQX and CGP 40116, respectively, and the $GABA_A$ and $GABA_B$ receptor mediated responses were inhibited by picrotoxin and CGP 55845A, respectively (Fig. 3.2 & Table 3.1).

Table 3.1. Antagonists used to inhibit ionotropic glutamate and GABA receptor-mediated responses.

Receptor	Response	Antagonist	Concentration Used
AMPA/Kainate	$EPSP_A$	NBQX	1–2 μM
		CNQX	10 μM
NMDA	$EPSP_N$	AP5	50 μM
		CGP 40116	50 μM
$GABA_A$	$IPSP_A$	Picrotoxin	50 μM
$GABA_B$	$IPSP_B$	CGP 55845A	1 μM
		CGP 35348	1 mM

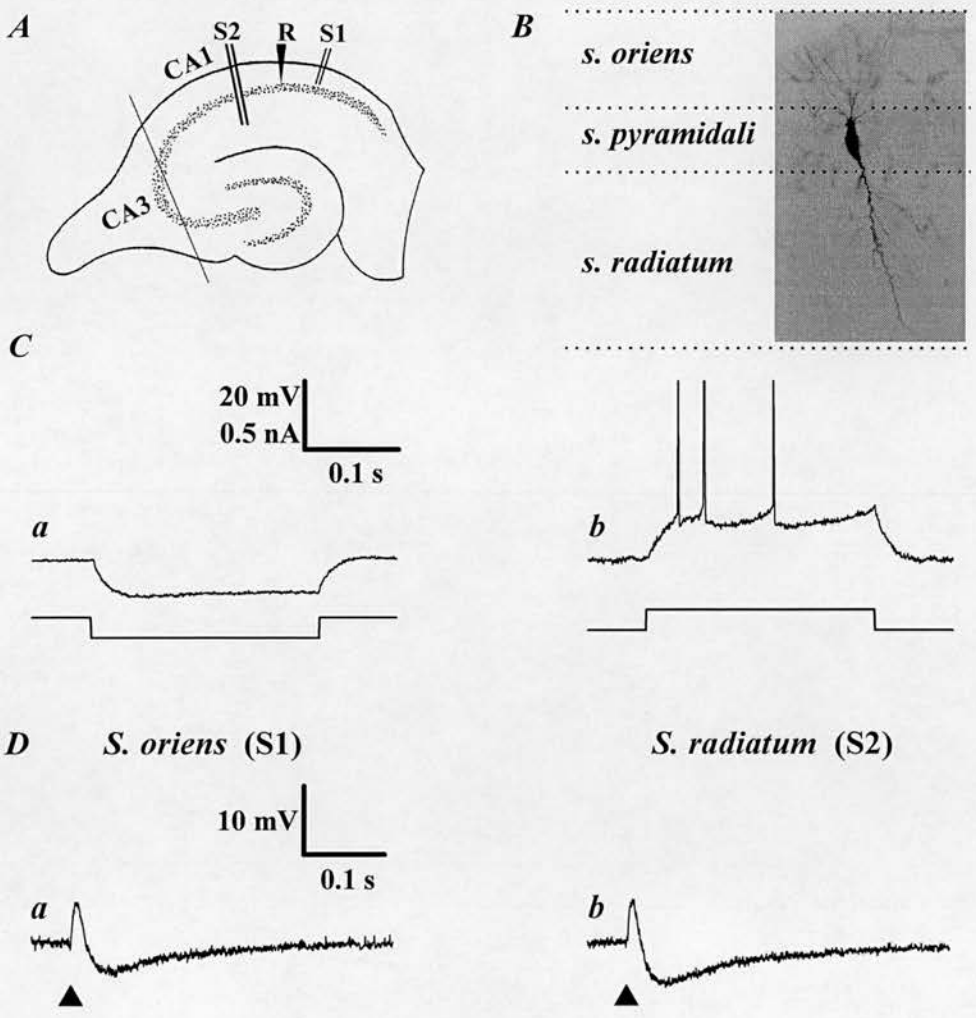

Figure 3.1. Characteristics of the passive membrane properties of CA1 pyramidal neurones and responses to stimulation of *stratum radiatum* and *stratum oriens*.

A, shows a schematic diagram of a rat hippocampal slice to illustrate the positioning of recording (R) and stimulating (S1 & S2) electrodes required to evoke the synaptic responses in *D*. B, is a light micrograph of a rat hippocampal CA1 pyramidal neurone stained by intracellular biocytin injection. C, shows voltage responses (upper traces) and current applied (lower traces) for negative (*a*) and positive (*b*) 0.3 nA current steps which result in hyperpolarizing (*a*) and depolarizing (*b*) voltage responses. Note that in *Cb* the cell depolarized to a potential where action potentials were evoked and that the frequency of action potential (spike) firing adapted with time (spike frequency adaptation). *Ca*, is an average of four successive records taken at the same membrane potential and *Cb*, is a single trace. The membrane potential of this neurone was -67 mV. *D*, shows intracellular recordings from a CA1 neurone in response to just suprathreshold single shock stimulation in the *s. oriens* (*a*) and *s. radiatum* (*b*). Note that a fast EPSP is followed by an IPSP in both responses. In this and subsequent figures, filled triangles below each synaptic trace indicate the time of afferent stimulation. The membrane potential of this neurone was -65 mV. In *D*, traces are averages of two records taken at the same membrane potential separated by a 30 s interval.

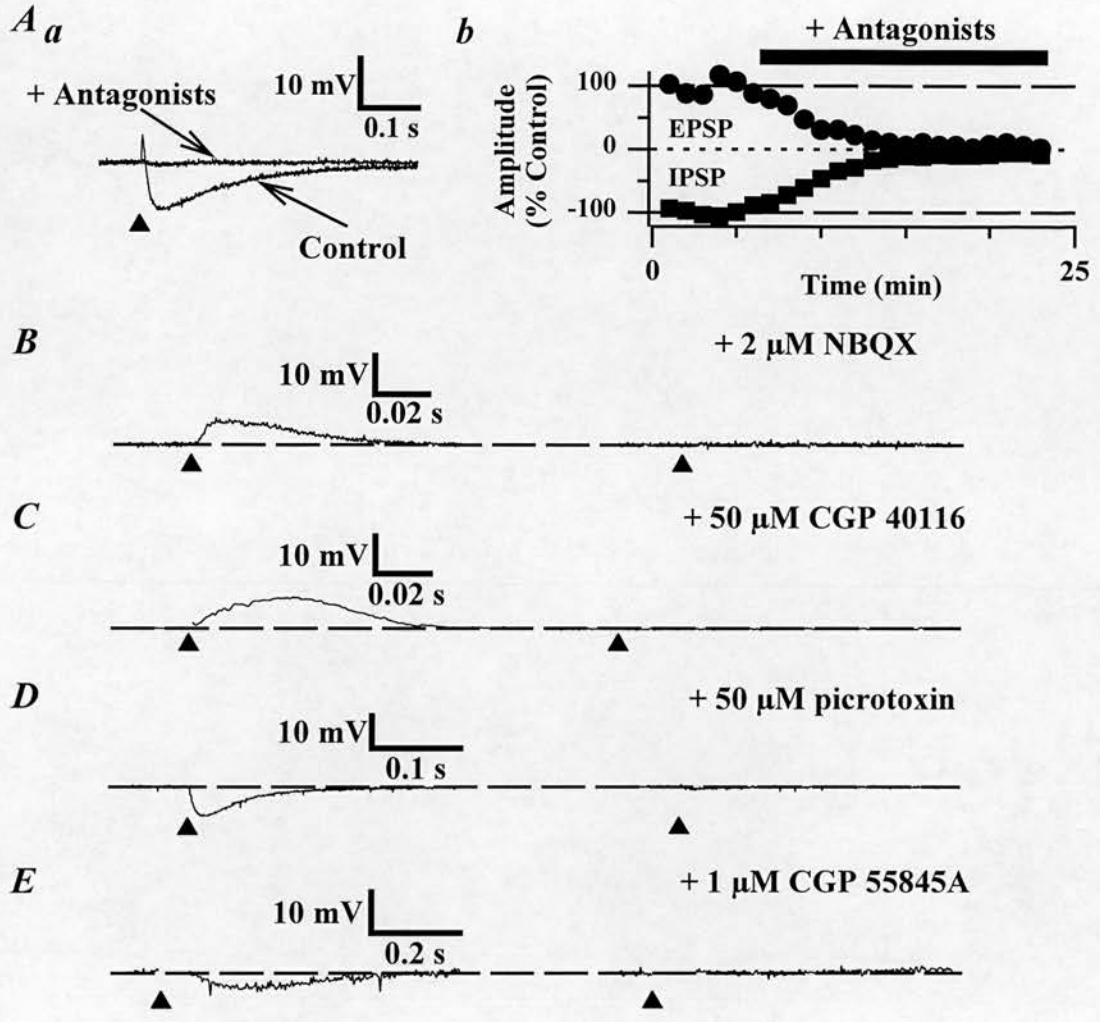

Figure 3.2. NBQX, CGP 40116, picrotoxin and CGP 55845A inhibit isolated AMPA, NMDA, $GABA_A$ and $GABA_B$ receptor-mediated synaptic responses, respectively.

Aa, shows superimposed intracellular recordings of responses to single shock stimulation of *s. oriens*. Note that in control medium (control), following the stimulus, a fast EPSP is followed by an IPSP and following the addition of NBQX (2 μM), CGP 40116 (50 μM), picrotoxin (50 μM) and CGP 55845A (1 μM) this response was abolished (+ Antagonists). *Ab*, plots the peak amplitudes of the successive EPSPs and IPSPs expressed as a percentage of the mean amplitude in control medium against time for a single experiment. The bar indicates the time during which the four antagonists listed in *A* were applied. The membrane potential of this neurone was -64 mV. *B–E* (*left hand recordings*), show recordings of isolated $EPSP_A$ (*B*) $EPSP_N$ (*C*), $IPSP_A$ (*D*) and $IPSP_B$s (*E*) (isolated using appropriate combinations of ionotropic glutamate and GABA receptor antagonists). The *right had recordings* in *B–E* (+ antagonist) show responses evoked in the additional presence of 2 μM NBQX (*B*), 50 μM CGP 40116 (*C*), 50 μM picrotoxin (*D*) or 1 μM CGP 55845A (*E*). Note that NBQX, CGP40116, picrotoxin and CGP 55845A completely inhibited the isolated EPSP/IPSPs. Also, note that, for illustrative purposes, different time scales were used for *D* and *E*. The membrane potentials for these neurones were -70 mV (*B*), -65 mV (*C*), -64 mV (*D*) and -64 mV (*E*).

Table 3.2 shows a summary of the kinetics of these responses. All components of this multi-component response were abolished by the combined application of these four antagonists (Fig. 3.2*A*). Table 3.1 summarizes the concentrations of antagonists used and the receptors and responses that are sensitive to each antagonist.

3.2.3. Stimulation Evoked Slow EPSPs

Increasing the stimulus intensity to 2–10 fold that which evoked a fast EPSP/IPSP evoked a much slower EPSP in 136 out of 139 neurones in which this was attempted (Fig. 3.3*Aa*). In a random group of neurones slow EPSPs which had a mean amplitude of 7.7 ± 0.4 mV had a time to peak of 2.1 ± 0.1 s and an overall duration of 24.3 ± 2.3 s ($n = 29$; Table 3.2 and Fig. 3.3*A*). The peak amplitude and duration of the EPSP were highly dependent upon the stimulus strength used and both parameters increased with increasing stimulus intensity (Fig. 3.3*Bb*). The EPSP often resulted in intense discharges of action potentials and in some neurones the EPSP depolarized the membrane beyond the action potential firing threshold to a region in which action potential generation was inactivated (Fig. 3.3*B*).

In order to investigate the effects of pharmacological compounds on the slow EPSP, it was necessary to decide on an optimum stimulation frequency for evoking this response. If two stimuli were applied to the *stratum oriens* (*s. oriens*) separated by 2 min, the response to the second stimulus was reduced in amplitude in 9 out of 14 cells (Fig. 3.4*A* and 3.5*C*). In contrast, if an interstimulus interval of 5–8 min was used, there was no significant reduction in the amplitude of the second response (Fig. 3.4*B* and 3.5*D*). In this respect, it was possible to obtain reproducible slow EPSPs at this stimulation frequency (Fig. 3.*4C*). As such, this protocol was used routinely in all experiments investigating the effects of drugs on the slow EPSP.

It seemed possible that a large first response might cause a greater depression of the second response due to an over-activation of the system, leading to, for example, a depletion of transmitter. However, further investigation of the depression of the EPSP revealed no clear correlation between the amplitude of the first response and the percentage depression of the second response although there was a tendency for the second response to be smaller when the first response was larger (Fig. 3.5*A–C*).

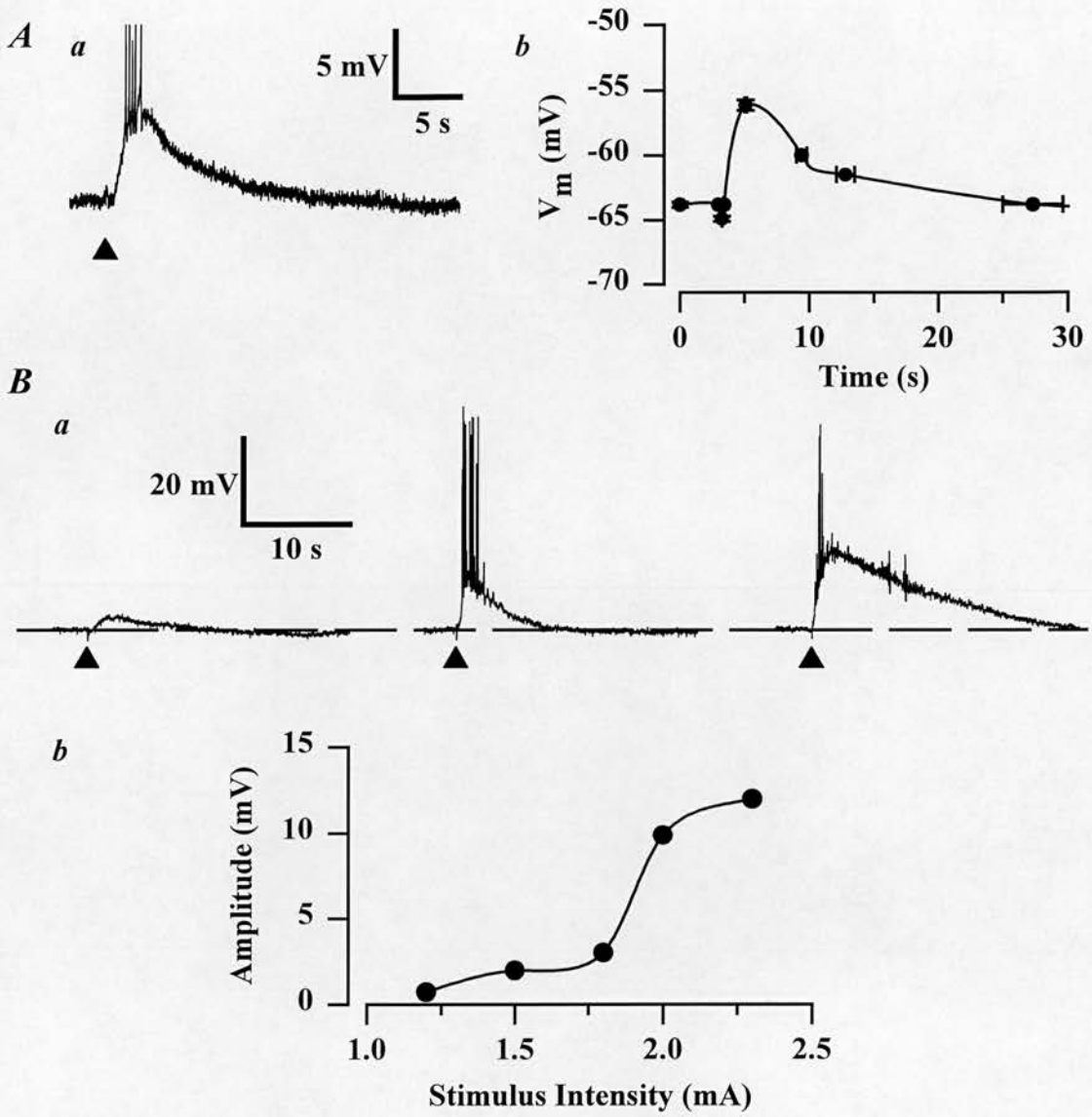

Figure 3.3. The time course and stimulus dependent properties of the slow EPSP.

Aa, is an intracellular recording of a response to a single stimulus of the *s. oriens*. *Ab*, is a plot of the time course of the slow EPSP using values generated from pooled data from 29 cells as listed in Table 3.2. The x coordinate plots the mean time points at which the start, peak, 50% recovery and 70 % recovery of the response occur. The y coordinate uses the mean holding potential of the 29 neurones as the baseline membrane potential and the mean amplitudes of the residual IPSP and slow EPSP. Values are means ± S.E.M. represented by the error bars. In *Ba*, the traces are single sweeps, which illustrate the stimulus-dependent activation of a slow EPSP in the presence of the four amino acid receptor antagonists. The stimulus intensities used to evoke the responses from left to right were 1.8, 2.3 and 4.0 mA respectively. Action potentials are truncated due to the low sampling rate used to capture the entire slow EPSP. The membrane potential of this neurone was -64 mV. *Bb*, shows a graph in which the stimulus intensity (in mA) is plotted against the amplitude of the resulting slow EPSP in mV. In this and subsequent figures, unless stated otherwise, traces are individual synaptic responses recorded intracellularly in response to a single stimulus delivered in the *s. oriens* in the presence of 1–2 μM NBQX, 50 μM CGP 40116, 50 μM picrotoxin and 1 μM CGP 55845A. Each sweep was taken at the same membrane potential achieved using DC injection to compensate for any drug-induced or spontaneous changes in membrane potential.

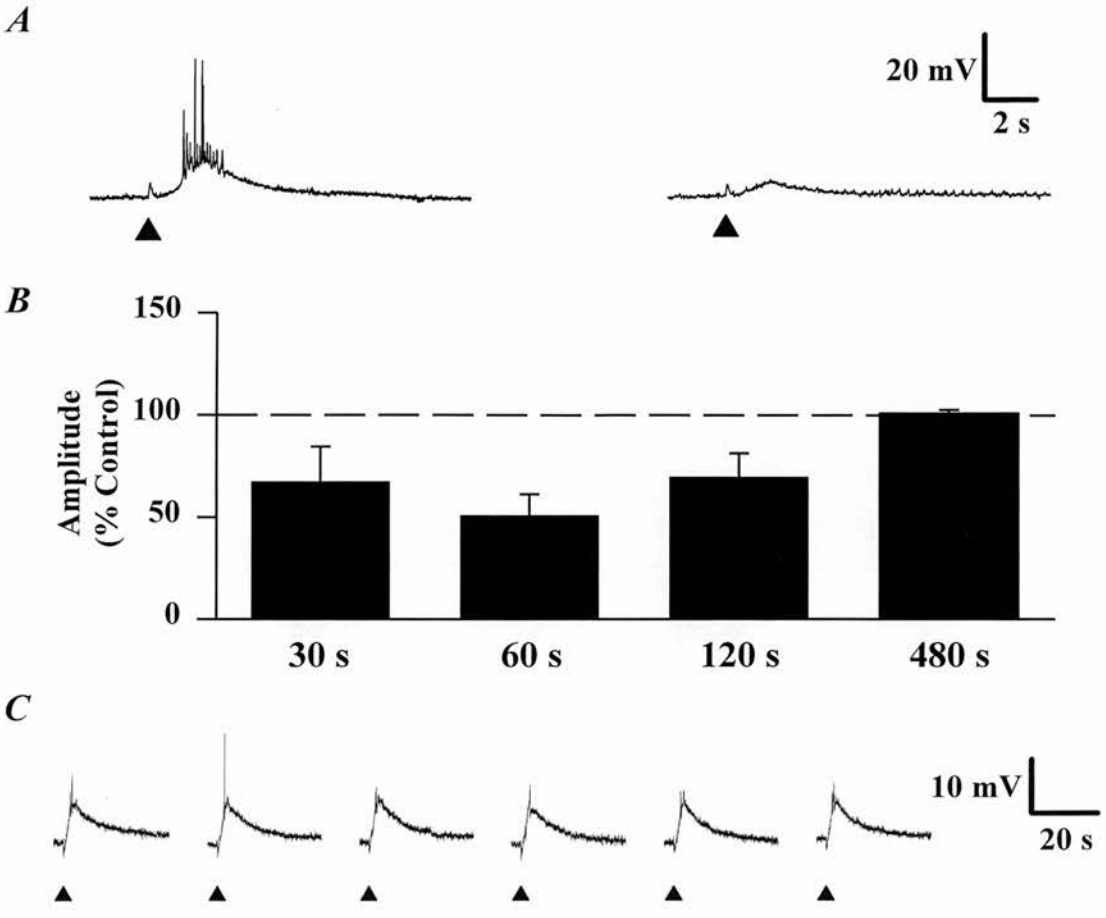

Figure 3.4. Depression of the slow EPSP with interstimulus intervals of less than 8 min.

A, shows two successive synaptic responses evoked by a single stimulus with an interstimulus interval of 120 s. Note that the second response is reduced in amplitude. The membrane potential of this neurone was -64 mV. *B*, is a bar graph representing the amplitude of a second EPSP when an interstimulus interval of 30, 60, 120 and 480 s was used. The amplitude of the second response is expressed as a percentage of the control response. Note that when an interstimulus interval of 30, 60 and 120 s was used, the amplitude of the second EPSP was depressed. In contrast, when an interstimulus interval of 480 s was used, there was no significant depression of the amplitude of the second response. *C*, shows six successive synaptic responses to single stimuli applied with an interstimulus interval of 480 s. Note that there is no clear depression of the amplitude of the EPSP with repeated stimulation. The membrane potential of this neurone is -64 mV.

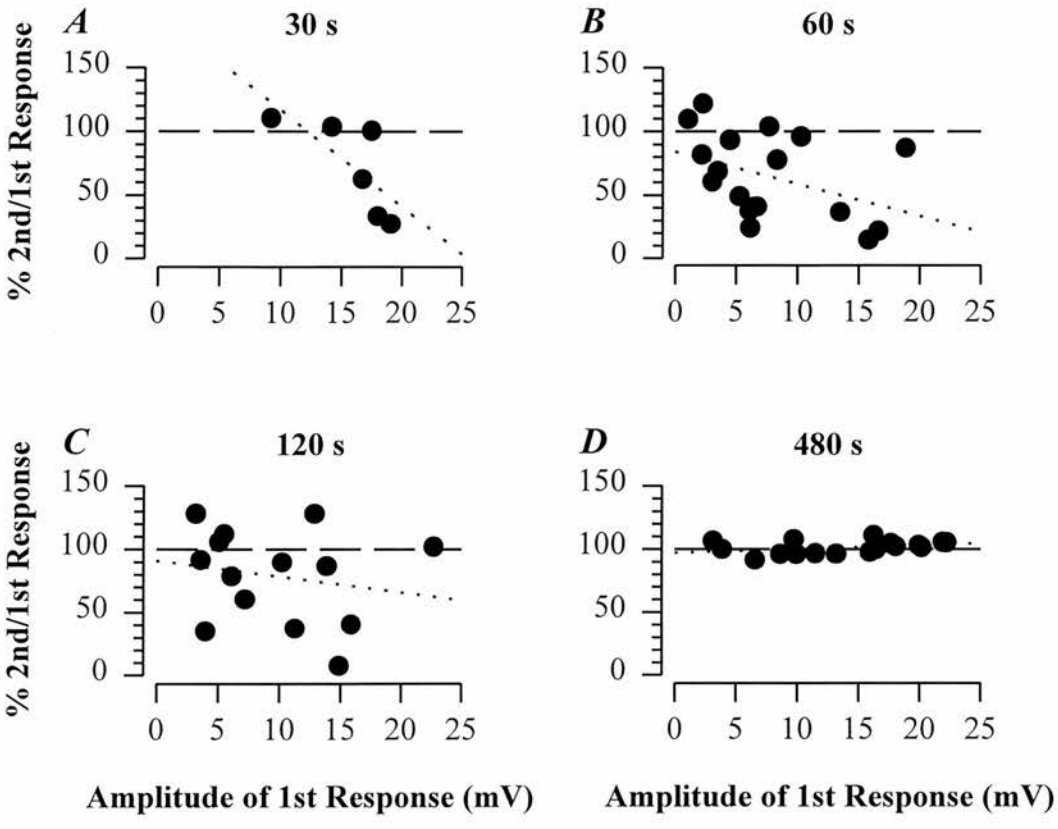

Figure 3.5. Relationship between the amount of depression of the slow EPSP with the amplitude of the first response with different interstimulus intervals.

Graphs *A–D*, plot the amplitude of a control (1st) EPSP on the abscissa with the percentage depression/potentiation of a second (2nd) response evoked following an interstimulus interval of 30 s (*A*), 60 s (*B*), 120 s (*C*) and 480 s (*D*). In *A-D*, the dashed line represents 100 % and the dotted line represents a regression line. Note that there was no clear correlation between the amplitude of the first response and the amount of depression of the second response at intervals of 30, 60 and 120 s (*A*, *B* and *C*) although there is a trend towards greater depression at larger amplitudes of the first response. Also note that at an interstimulus interval of 480 s (*D*) there was no depression of the second response. Each point, in one particular graph, represents responses evoked in a different neurone.

As mentioned above, in a small proportion of neurones, there was little or no depression of the slow EPSP when the cell was stimulated every 30–120 s (Fig. 3.5*A–C*).

Table 3.2 Kinetics of synaptic responses.

Response	Time to peak	Duration	*n*
$EPSP_M$	2.1 ± 0.1 s	24.3 ± 2.3 s	$n = 29$
$EPSP_A$	10.3 ± 0.8 ms	72.2 ± 3.0 ms	$n = 5$
$IPSP_A$*	18 ± 1 ms	227 ± 3 ms	$n = 3$*
$IPSP_B$*	141 ± 3 ms	824 ± 153 ms	$n = 7$*

* Data provided by C. H. Davies.

3.2.4. PHARMACOLOGY OF THE SLOW EPSP

The effects of the AChR agonist CCh were compared with the effects of stimulation of the *s. oriens*. CCh (3 μM), when bath applied for 30–60 s, caused a depolarization which lasted a matter of minutes and was reversed following washout ($n = 7$; Fig. 3.6*A*). This CCh-induced depolarization was associated with an increase in input resistance as calculated from the membrane voltage responses to negative current steps recorded at the same membrane potential (Fig. 3.6*A*). In a similar manner, the slow EPSP was associated with an increase in input resistance ($n = 3$; Fig. 3.6*B*).

As illustrated in Fig. 3.7, the slow EPSP was not affected significantly by the metabotropic glutamate receptor (mGluR) antagonist (+)-MCPG (1000 μM, $n = 3$, Fig. 3.7*A*). Addition of the acetylcholinesterase (AChE) inhibitor physostigmine (1–5 μM), however, caused a large enhancement of both the peak amplitude and duration of the slow EPSP ($n = 5$; Fig. 3.7*B&C*). In contrast, the mAChR antagonists atropine (2–5 μM) and AFDX 116 (1 μM) abolished or reduced the slow EPSP ($n = 7$ & $n = 5$; Fig. 3.8). In three cells, atropine (2–5 μM) was also effective at inhibiting responses evoked in the presence of physostigmine (1–5 μM), such that responses in the combined presence of physostigmine and atropine were 10.1 ± 8.1 % of mean control responses in the presence of physostigmine alone (data not shown).

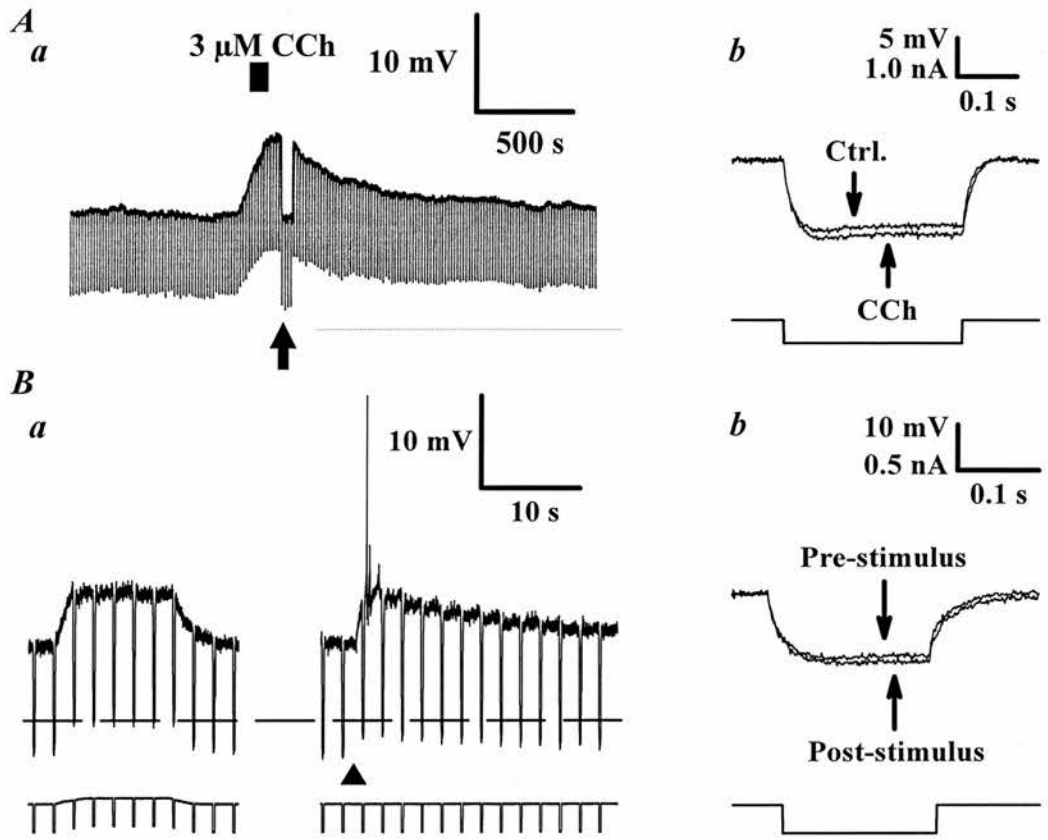

Figure 3.6. A comparison of the effects of carbachol and cholinergic afferent stimulation.

A, shows the effect of carbachol (CCh; 3 μM) on the membrane potential and input resistance of a neurone. The trace is a chart record of membrane potential and cell input resistance. DC injection was kept constant except when the increase in input resistance evoked by CCh was measured at the time point indicated by the arrow. The bar above the chart record represents the time for which CCh was applied. The initial membrane potential of this neurone was -64 mV. *Ab*, shows a comparison of the hyperpolarizing responses in control (Ctrl.) and following application of CCh on an expanded time scale. The input resistance was measured from this change in membrane potential in response to hyperpolarizing current steps of -0.3 nA applied for 0.2 s every 2.0 s. In *Ba* the cell membrane potential was manually clamped at -59 mV before an $EPSP_M$ was evoked so that the voltage response to the hyperpolarizing current steps could be compared with that evoked at the peak of the $EPSP_M$ (also at a V_m of -59 mV). *Bb*, shows a comparison of these responses on an expanded time scale. The current injected into the cell is shown below the voltage traces. The initial membrane potential of this cell was -64 mV. The filled triangles below each synaptic trace indicate the time of afferent stimulation. Note that both CCh and stimulation of the *s. oriens* caused a depolarization and increase in input resistance.

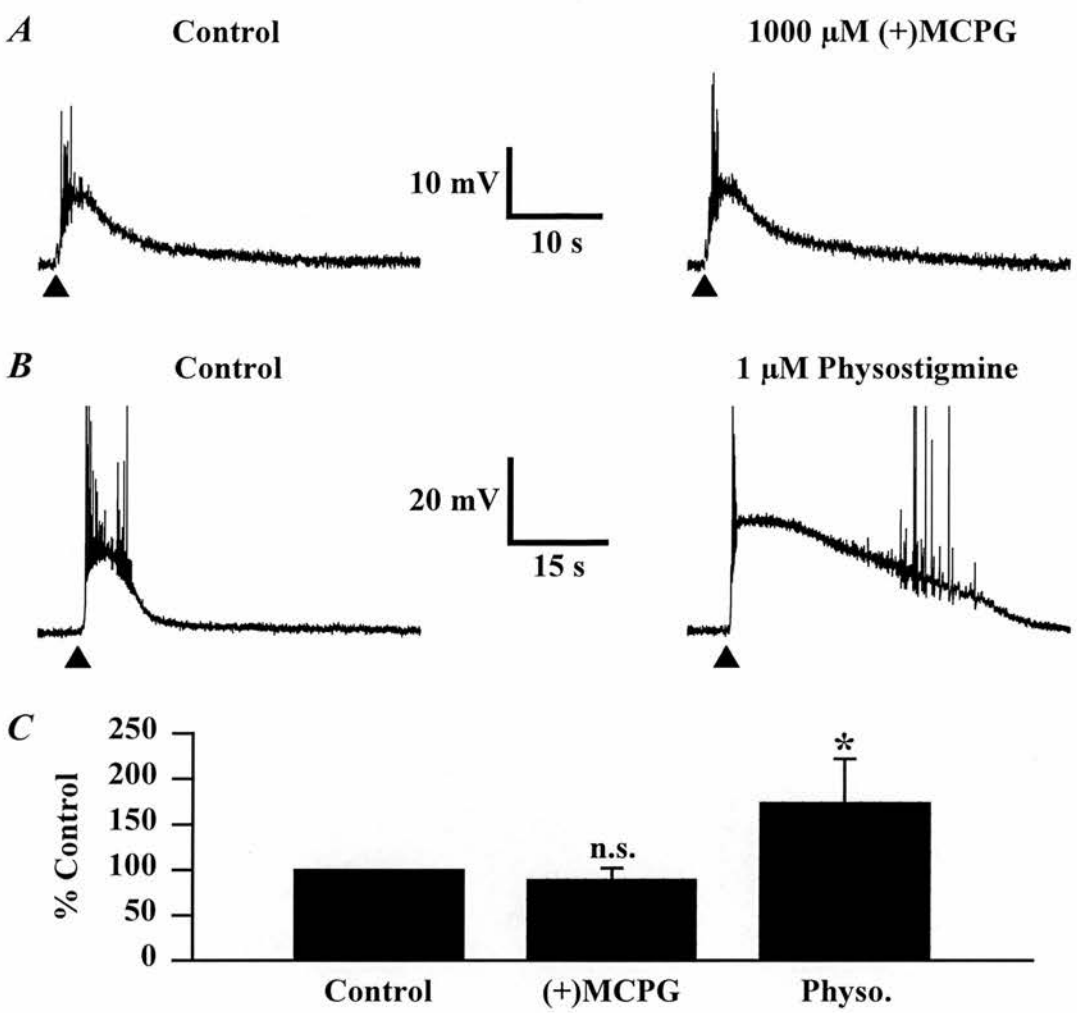

Figure 3.7. The effects of (+)-MCPG and physostigmine on the slow EPSP.

In *A*, and *B* synaptic traces are single sweeps illustrating the effects of 1000 μM (+)-MCPG (*A*) and 2 μM physostigmine (*B*) on slow EPSPs evoked in two separate neurones. The membrane potentials of these cells were -63 mV and -64 mV respectively. *C*, is a bar graph in which the pooled data for the peak amplitude of $EPSP_M$s recorded after 15–30 minutes in the presence of either (+)-MCPG (1000 μM; $n = 3$) or physostigmine (2 μM; $n = 3$) is expressed as a percentage of the mean value of the control $EPSP_M$s recorded over a 20–30 min baseline period prior to each drug application. Values are means ± S.E.M. such that 100 % is equivalent to no change. Note that whilst (+)-MCPG had no significant effect on the slow EPSP ($P > 0.05$) physostigmine significantly enhanced the slow EPSP ($P < 0.05$). In this and all subsequent figures statistical significance is represented by an asterisk and n.s. represents values that were not significantly different from control values ($P > 0.05$).

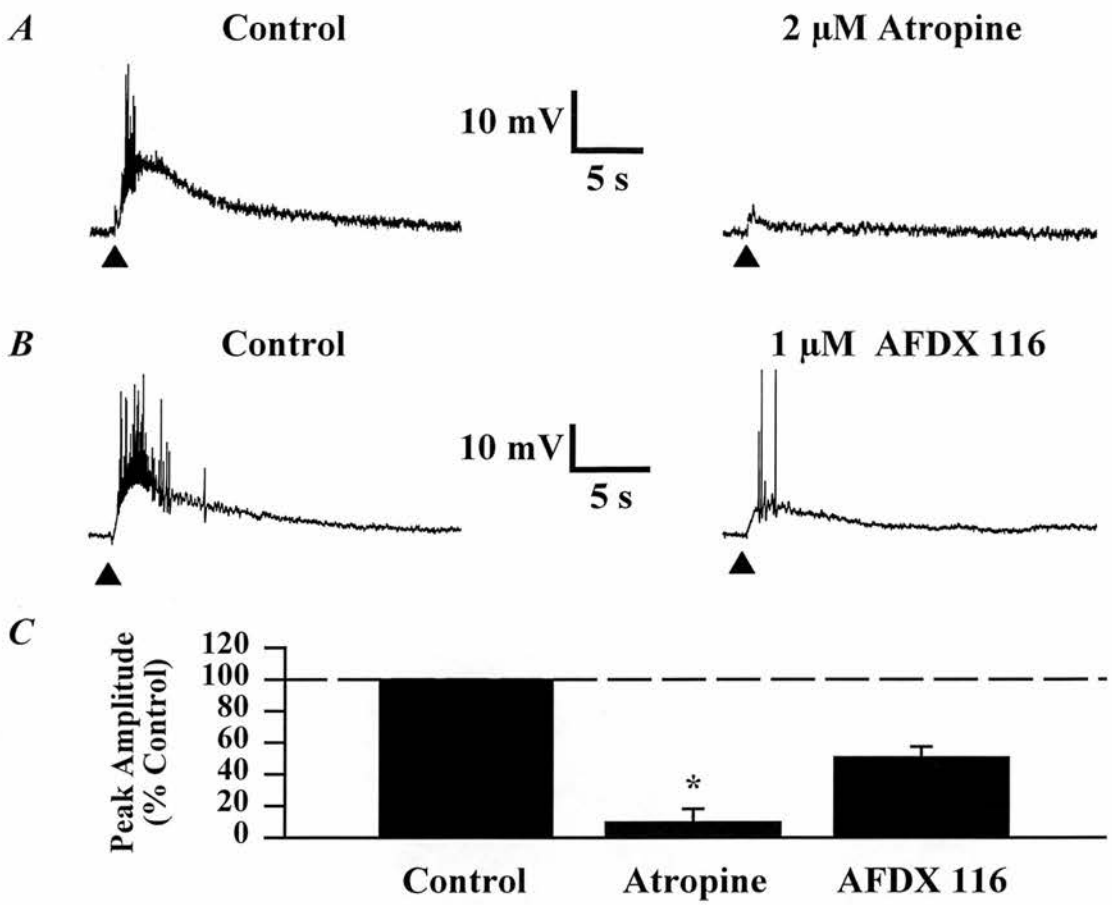

Figure 3.8. The effects of atropine and AFDX 116 on the slow EPSP.

In *A*, and *B* traces are single sweeps illustrating the effects of 2 μM atropine (*A*) and 1 μM AFDX 116 (*B*) on slow EPSPs evoked in two separate neurones. The membrane potentials of these cells were -63 and -64 mV, respectively. *C*, is a bar graph in which the pooled data for the peak amplitude of $EPSP_M$s recorded after 15–30 minutes in the presence of either 2 μM atropine ($n = 3$) or 1 μM AFDX 116 ($n = 3$) is expressed as a percentage of the mean value of the control $EPSP_M$s recorded over a 20–30 min baseline period prior to each drug application. Values are means ± S.E.M. such that 100 % is equivalent to no change. Note that atropine significantly depressed $EPSP_M$s ($P < 0.05$) and AFDX 116 depressed the slow EPSP by approximately 50 %, although this was not statistically significant ($P > 0.05$).

These results are entirely consistent with the slow EPSP being mediated by activation of mAChRs and, as such, this slow EPSP will be referred to as a mAChR-mediated EPSP ($EPSP_M$).

Cholinergic (and GABAergic) systems have been linked to the synchronization of θ-frequency oscillations in the hippocampus (Stewart & Fox, 1990). In some cells, the response to CCh depolarized the cell past the firing threshold for action potential generation, causing the cell to fire action potentials at a mean frequency of 4–8 Hz (Fig. 3.9*A*). Interestingly, analysis of the decaying phase of $EPSP_M$s also showed membrane potential oscillations in the frequency range of 4–8 Hz (Fig. 3.9*B*).

3.2.5. Inhibition of Spike Frequency Adaptation

Physostigmine (1 μM), as well as causing an increase in the amplitude of the $EPSP_M$, also caused a reduction in SFA as measured by the number of action potentials (spikes) fired in response to a 300 ms long +0.15–0.30 nA current step ($n = 6$; Fig. 3.10*A&C*). This effect presumably occurred as a result of raising extracellular levels of spontaneously released ACh due to inhibition of AChE activity by physostigmine (Cole & Nicoll, 1984a; Azouz *et al.* 1994). A similar inhibition of SFA has been reported on stimulation of the *s. oriens* with 10 stimuli at 100 Hz in the presence of physostigmine (Cole & Nicoll, 1984a). Here, stimulation of the *s. oriens* with a single stimulus at an intensity subthreshold for evoking an $EPSP_M$, and in the absence of physostigmine, also resulted in an inhibition of SFA (Fig. 3.10*B*). As such, in 7 cells, there was a significant increase in the number of spikes fired in response to a depolarizing step applied 2 s after a stimulus (post-stimulus) when compared with a step applied 2 s before the stimulus (pre-stimulus) ($P < 0.05$; students t-test; Fig. 3.10*D*). In contrast, when two steps were applied in the absence of a stimulus, there was no significant difference in the number of spikes fired during the first or second step ($n = 7$; Fig. 3.10*Ba&D*).

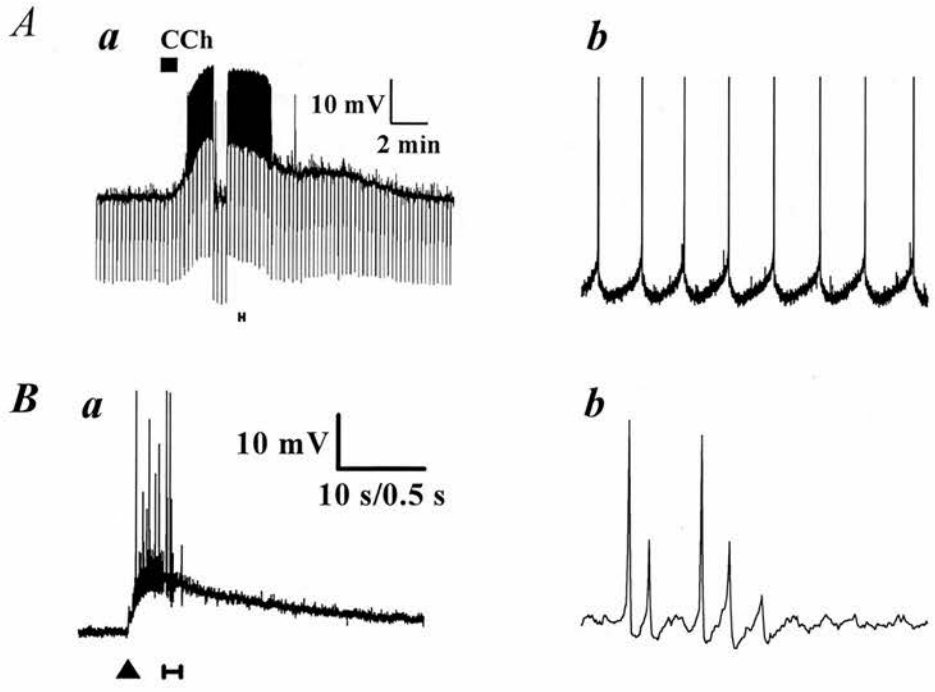

Figure 3.9. A comparison of the membrane oscillations produced by muscarinic receptor activation by CCh and cholinergic afferent stimulation.

A, shows the effect of carbachol (CCh; 3 μM) on the membrane potential and input resistance of a neurone. The trace is a chart record of membrane potential and cell input resistance. DC injection was kept constant except when the increase in input resistance evoked by CCh was measured. The bar above the chart record represents the time for which CCh was applied. The initial membrane potential of this neurone was -64 mV. *Ab*, shows an expanded time scale of the same response from the time point indicated by the line below *Aa*. Note that, in the presence of CCh, the neurone fires regularly at a rate of approximately 4 Hz. *Ba* is a $EPSP_M$, evoked using a single stimulus as previously described. *Bb*, shows an expanded time scale of the same response from the time point indicated by the line below *Bb*. Note that, in the during the falling phase of the $EPSP_M$, the neurone fires, and later oscillates regularly at a rate of approximately 6 Hz. The membrane potential of this cell was -64 mV. Note that both CCh and stimulation of the *s. oriens* caused a depolarization and induced repetitive firing/oscillations in the frequency range 4-8 Hz.

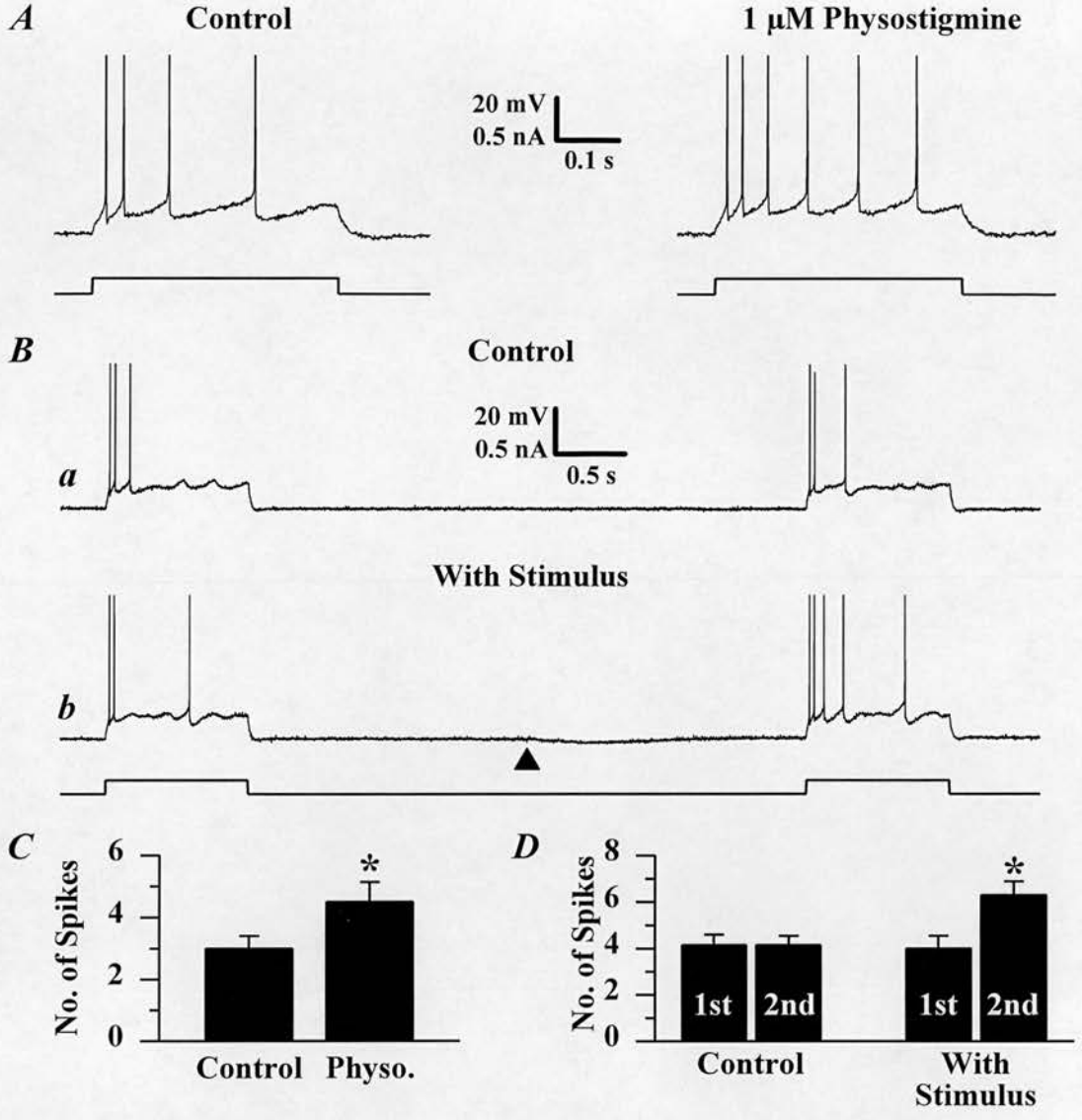

Figure 3.10. Inhibition of spike frequency adaptation by endogenous ACh.

A, shows responses evoked by a depolarizing current step (+0.15 nA, 400 ms) in control medium and in the presence of physostigmine (1 μM). The membrane potential of the cell was maintained at -63 mV throughout this experiment by injecting DC through the recording electrode. In *B*, traces are continuous records of the membrane potential of a single cell in which depolarizing current steps (+0.15 nA, 1000 ms) were delivered twice at fixed time points without (*a*) and with a stimulus (*b*) applied at the time point indicated by the filled triangle. The stimulation was delivered at an intensity just subthreshold for activating an $EPSP_M$. The responses were obtained in medium containing the four ionotropic glutamate and GABA receptor antagonists. The membrane potential of the neurone was -64 mV. The bar graph in *C*, shows pooled data for the effect of physostigmine (1 μM; $n = 4$). The mean number of spikes fired in response to depolarizing current steps are plotted in control medium and in the presence of physostigmine. Note that there was a significant increase in the number of spikes fired in the presence of physostigmine. The bar graph in *D*, shows pooled data ($n = 7$) for the number of spikes fired during two current steps (1st and 2nd) like those represented in *B*, with no intervening stimulus (Control) and with an intervening stimulus (With Stimulus). Note that there was no significant difference between the number of spikes fired in response to the first and second step in control ($P > 0.05$) but there was a significant increase when a stimulus was applied ($P < 0.05$). Both physostigmine and cholinergic afferent stimulation reduced SFA.

3.2.6. Pharmacology of Stimulation Evoked Inhibition of Spike Frequency Adaptation

The pharmacology of the receptor mediating this inhibition of SFA was also investigated. To do this CCh (0.5 μM) was used as, when applied to the perfusing ACSF, it also caused an inhibition of SFA which was subsequently reversed following washout ($n = 4$; Fig. 3.11*B&D*). This CCh-induced inhibition of SFA was inhibited by atropine (1 μM; $n = 2$; data not shown); results that are consistent with previous studies using both CCh and ACh as agonists (Ben Ari *et al.* 1981, Cole & Nicoll, 1983; 1984; Azouz *et al.* 1994).

Likewise, inhibition of SFA evoked by pathway stimulation, in the presence of ionotropic glutamate and GABA receptor antagonists, was inhibited by the mAChR antagonist atropine (1 μM). As such, in the presence of the antagonist there was no significant difference in the number of spikes fired pre- and post-stimulus ($n = 3$; $P > 0.05$, students t-test; Fig 3.11*A*). Taken together with the results using agonist application, these results are consistent with action of mAChRs to inhibit SFA and furthermore that synaptically released ACh is capable of reproducing this effect in the absence of AChE inhibitors.

3.2.7. Action Potential Amplitude and Duration

Stimulation of mAChRs by CCh prolongs the duration and reduces the amplitude of action potentials in the hippocampus (Figenschou *et al.* 1996). Therefore, a preliminary study was carried out to investigate the effect of cholinergic afferent stimulation in *s. oriens* on action potential amplitude and duration. In two neurones, single action potentials were evoked by applying 50 ms depolarizing current steps to the neurone every 1 s and the resulting action potentials compared before and after stimulation in *s. oriens*. In one neurone, a 3 mA stimulus did not alter the shape of action potentials evoked following the stimulus. However, at a stimulus intensity of 3.5 mA the duration of the action potentials following stimulation was consistently greater than the mean duration before the stimulus (Fig. 3.12). The amplitude of the action potential was not altered.

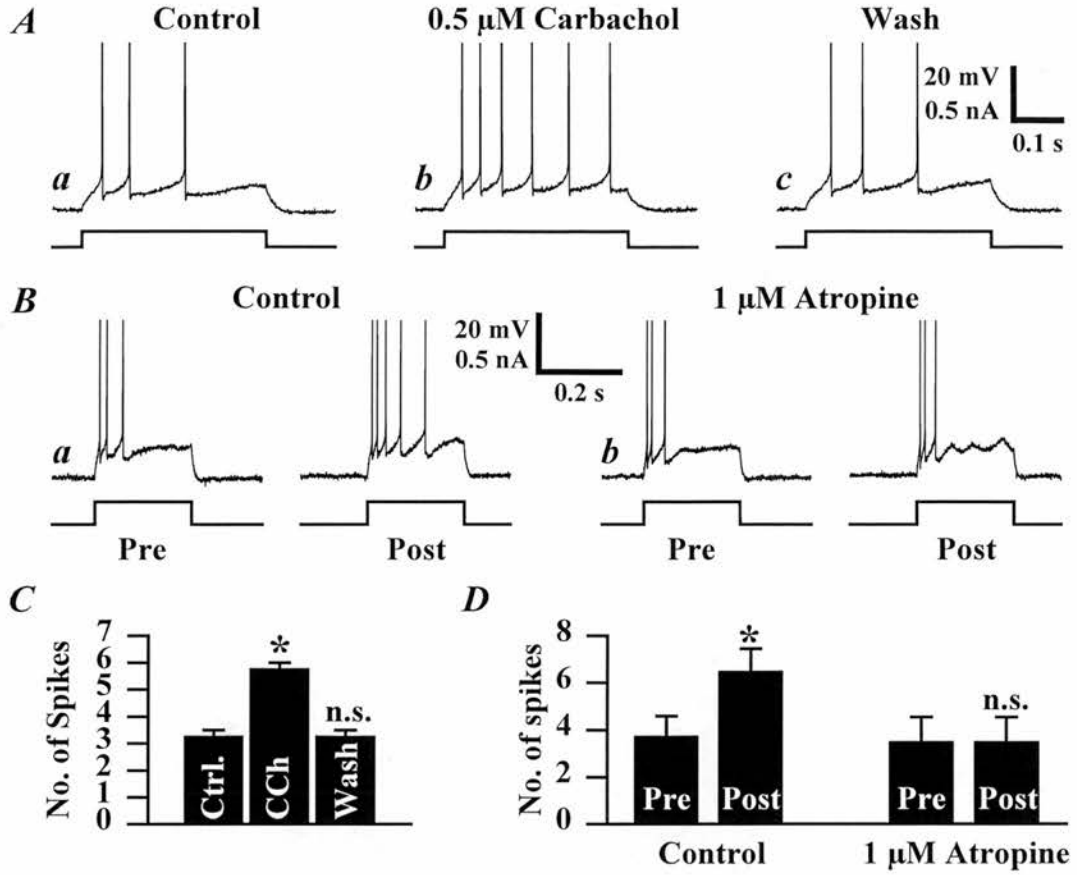

Figure 3.11. A comparison of the effects of carbachol and cholinergic afferent stimulation on spike frequency adaptation.

A, shows, from left to right, responses evoked by a depolarizing current step (+0.2 nA, 400 ms) in control medium, in the presence of CCh (0.5 µM) and following washout. The membrane potential of the neurone was maintained at -66 mV throughout this experiment as described in Figure 3.3. In *B*, traces are generated as described in Figure 3.10 but using 0.2 nA steps. The responses were obtained in medium containing the four ionotropic glutamate and GABA receptor antagonists (*a*) and in the additional presence of 1 µM atropine (*b*). The point of pathway stimulation occurs during the break in each of *Ba* and *Bb*. Note that in control medium, SFA was reduced following pathway stimulation (*Ba*) and this reduction was abolished by atropine (*Bb*). The membrane potential of the neurone was maintained at -65 mV throughout this experiment. In *A* and *B*, current applied is represented below each voltage trace. The bar graph in *C*, shows pooled data for the effect of CCh (0.5 µM; $n = 3$). The mean number of spikes fired in response to a depolarizing current step is plotted in control medium (Ctrl.) in the presence of CCh (0.5 µM) and following washout (Wash). Note that CCh significantly increased the number of spikes fired ($P < 0.05$) and that the number of spikes fired following washout of CCh was not significantly different from those fired in control ($P > 0.05$). The bar graph in *D*, shows pooled data ($n = 4$) for the number of spikes fired in response to current steps evoked before (Pre) and following a stimulus (Post) in control medium and following the addition of atropine (1 µM). Note there was a significant increase in the number of spikes fired in control ($P < 0.05$) but there was no significant increase in the presence of atropine ($P > 0.05$).

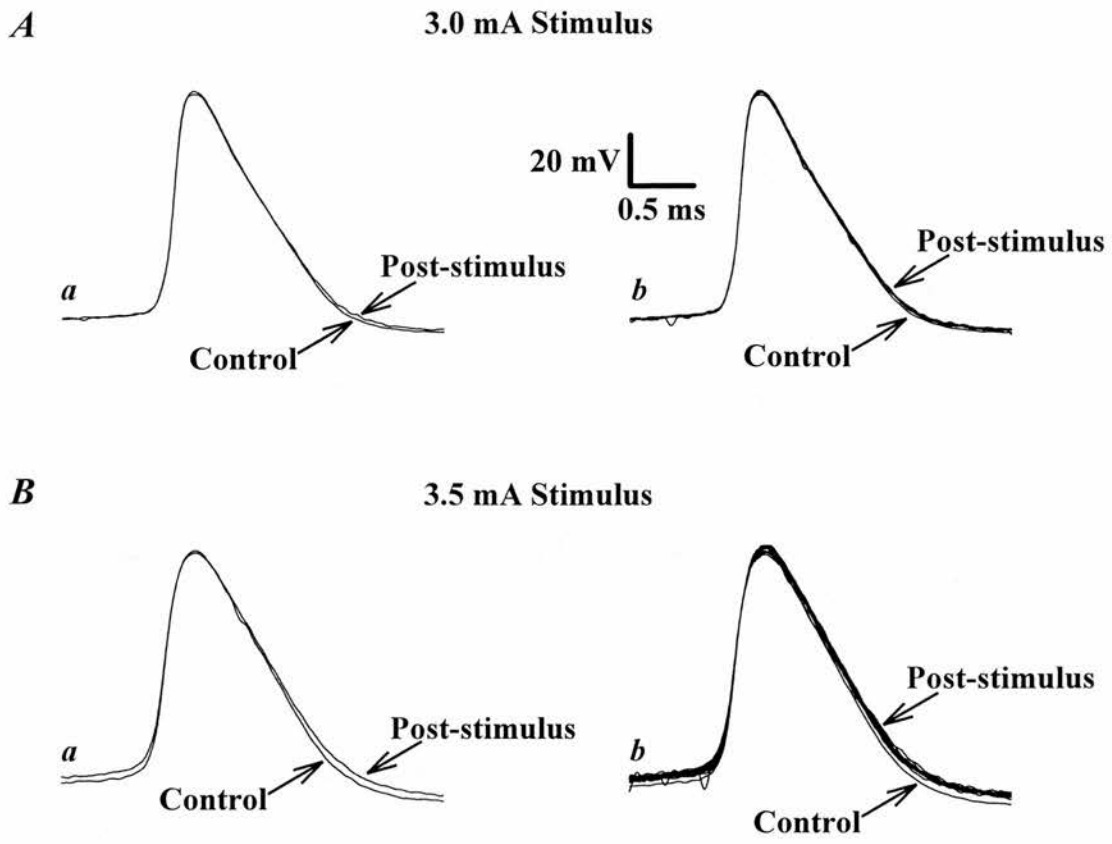

Figure 3.12. The effects of cholinergic afferent stimulation on action potential kinetics.

In *A* & *B*, synaptic traces are action potentials evoked by 50 ms positive current steps before (Control) and following a single stimulus of the *s. oriens* (Post-stimulus). In *A*, a stimulus of 3.0 mA was applied and in *B*, a stimulus of 3.5 mA was applied. In *Aa* and *Ba*, control action potentials are means of the 4 action potentials evoked prior to the stimulus and Post-stimulus action potentials are the means of 4 action potentials evoked following the stimulus. In *Ab* and *Bb*, controls are the same as the means in *Aa* and *Ba*, but individual post-stimulus action potentials are plotted to show the consistency of any change in the shape of the action potential. Note that in *A*, at the lower stimulus intensity there was no obvious change in the shape of action potentials evoked following the stimulus. In contrast, in *B*, at a slightly higher stimulus intensity there was a consistent lengthening in the duration of the action potentials evoked following the stimulus.

3.3. DISCUSSION

3.3.1. THE SLOW EPSP

Slow EPSPs mediated by mAChRs have been recorded previously from CA1 pyramidal neurones most commonly in response to high frequency bursts of stimuli e.g. 20–100 Hz for 0.5–1.0 s (Cole & Nicoll, 1984a; Madison *et al.* 1987; Segal, 1988; Pitler & Alger, 1990; Potier *et al.* 1992; Azouz *et al.* 1994). These EPSPs were often evoked in the presence of AChE inhibitors, which elevate the effective synaptic concentration of ACh. In addition, ionotropic glutamate and GABA receptor antagonists were not added to the perfusing medium and, therefore, mAChR-mediated EPSPs were superimposed on multiple glutamate- and GABA-mediated synaptic potentials. Results presented in this chapter have shown that a slow EPSP can be evoked by a single stimulus in the presence of ionotropic glutamate and GABA receptor antagonists without the need for AChE inhibitors. The waveform of this slow EPSP varied considerably between neurones but these differences could not be readily correlated with differences in other electrophysiological characteristics of the neurones in which they were recorded.

This slow EPSP was mediated by activation of mAChRs since it was blocked by the mAChR antagonists atropine and AFDX 116 and unaffected by the mGluR antagonist (+)-MCPG. In addition, the slow EPSP and accompanying increase in input resistance were mimicked by brief bath application of CCh. As such, the slow EPSP can be defined as a mAChR-mediated EPSP ($EPSP_M$). It is unclear, however, which subtype of mAChR mediates this $EPSP_M$ as the effects of only two antagonists were tested. Pitler & Alger (1990) classified the mAChR underlying the mAChR-mediated slow EPSP as an M_3 receptor. In the study by Pitler & Alger (1990), AFDX 116 inhibited slow EPSPs evoked by high frequency stimulation with an IC_{50} of 24 μM. In the present study, 10 μM AFDX 116 depressed $EPSP_M$s by approximately 50 %, although a full concentration response relationship was not constructed to obtain an IC_{50}. As such, AFDX 116 would appear to be slightly more potent in the present study compared with the earlier study (Pitler & Alger, 1990). This small difference may be explained by the different methods used to evoke the

responses (i.e. the use of mature male rats rather than young females, the absence of amino acid antagonists or the use of high frequency trains of stimuli). A more detailed analysis of the effects of a variety of mAChR antagonists will be required to confirm the subtype of mAChR underlying the $EPSP_M$, although this may require the development of more selective mAChR subtype-selective antagonists or transgenic animals deficient in specific mAChR subtypes.

As shown in previous studies, both the $EPSP_M$ and the depolarizing response to CCh were associated with an increase in input resistance (Cole & Nicoll, 1984a). This is consistent with the $EPSP_M$ being mediated by an inhibition of a K^+ leak current ($I_{K(LEAK)}$) (Fig. 3.13; Cole & Nicoll, 1984a; Madison *et al.* 1987).

One notable difference, however, between the $EPSP_M$ recorded in this study and slow EPSPs recorded previously was its large magnitude, even when evoked using just a single stimulus. The reason for this is unclear but it is possible that the ionotropic glutamate and GABA receptor antagonists relieve an inhibitory influence on the activation of this synaptic potential (See Chapter 6 for the effect of $GABA_B$ receptor activation on $EPSP_M$s). Alternatively, in some cells a number of voltage activated conductances may boost the apparent magnitude of the $EPSP_M$ (Fraser & McVicar, 1996). Preliminary observations, however, suggest that this is not the case, as the Ca^{++} channel antagonist nitrendipine does not significantly affect $EPSP_M$s (data not shown).

Slow EPSPs, evoked using high frequency stimulation, have been shown to be reduced in aged animals compared to young animals (Potier *et al.* 1992). In this respect, the age of the animals (2-5 weeks of age) may affect the expression of the mAChR-mediated responses. In addition, M_2 mAChRs are thought to mediate presynaptic autoinhibition of ACh release. M_2 mAChR densities and mAChR-mediated inhibition of field recorded glutamate EPSPs in the hippocampus have been shown to be reduced in animals younger than 5 weeks of age in comparison to adult animals (> 5 weeks) (Aubert *et al.* 1996; Milburn & Prince, 1993). As such, large magnitude of $EPSP_M$s seen in this study may due to a lower level of autoinhibition in the age of animals used compared to animals greater than 5 weeks of age.

Whatever the reason for the large amplitude of the $EPSP_M$s, the response kinetics were, in general, similar to those reported for slow EPSPs mediated by either mAChRs (Cole & Nicoll, 1984a; Pitler & Alger, 1990) as well as pure spontaneous and evoked mGluR-mediated EPSPs (Charpak & Gähwiler, 1991; Bianchi & Wong, 1995).

3.3.2. Fatigue of The Slow EPSP

A feature of the $EPSP_M$ that hampered the ease of its characterization was the ability to evoke it reproducibly only every 5–10 min. As such, relatively long intracellular impalements were required to obtain meaningful data. This contrasts with mGluR-mediated EPSPs which can be evoked every 30–60 s (Gerber *et al.* 1993). The reason(s) for the protracted fatigue of the $EPSP_M$ is unclear but by comparison with previous studies it appears to be independent of the stimulation protocol used to evoke it i.e. a high frequency train *versus* single stimulus (Cole & Nicoll, 1984a; Pitler & Alger, 1990). Preliminary observations of the profile of depression suggests that there is less depression of $EPSP_M$s evoked 15–30 s after the previous response than, for example, 60–120 s after the first response. This may suggest that there are multiple mechanisms underlying the fatigue of the response. Indeed in some neurones a depression was not observed even when a stimulus was reapplied 120 s after the original stimulus. However, it is clear that this fatigue is not caused by activation of AMPA, kainate, NMDA, $GABA_A$ or $GABA_B$ receptors because these experiments were carried out in the presence of antagonists of these receptors. However, a modulation by some other released neuromodulator (such as adenosine; see chapters 4 & 5) cannot be ruled out. Further experimentation will be required to ascertain the extent to which, for example, desensitization, transmitter depletion or shifts in ionic conductances might contribute to the depression.

Some $EPSP_M$s were followed by a pronounced hyperpolarization (1–3 mV) which may have been due to activation of a Ca^{++} activated K^+ conductance $I_{K(Ca)}$ by the strong depolarization of the neurone beyond the threshold for action potential firing (For review see Sah, 1996). The long duration of these hyperpolarizations (up to 6 min), however, suggests longer-term effects on cellular physiology. The amount of

depression is not clearly proportional to the amplitude of the original response, at least at amplitudes above 3 mV. This may suggest that it is merely the activation of the $EPSP_M$ and not the amount of activation that underlies the fatiguing of the $EPSP_M$. It is not clear, however, whether mAChR-mediated inhibition of SFA is subject to the same fatigue. Preliminary results suggest that it is possible to evoke an inhibition of SFA reproducibly by stimulating at higher rates than those used to evoke $EPSP_M$s. This may reflect the much lower level of activation required to evoke inhibition of SFA, which is insufficient to activate the mechanism underlying the fatigue of $EPSP_M$s.

A rapid desensitization of mAChR-mediated K^+ current in atrial myocytes has been reported and has been shown to be mediated predominantly through a G-protein-dependent phosphorylation of mAChRs (Shui *et al.* 1995). Relevant to the hippocampus, it has recently been shown that in CA3 neurones, a G-protein-mediated desensitization of a mAChR-mediated nonselective cation conductance can occur (Guérineau *et al.* 1997). It remains to be elucidated whether desensitization of mAChRs can account for the depression of $EPSP_M$s during repetitive stimulation.

3.3.3. Stimulation Evoked Inhibition of Spike Frequency Adaptation

As with slow EPSPs evoked in previous studies (Cole & Nicoll, 1984a; Segal, 1988; Azouz *et al.* 1994), stimulation that was sub-threshold for activating an $EPSP_M$ caused a reduction in SFA. This effect was mimicked by the AChR agonist CCh, an effect which is mediated by mAChRs (Cole & Nicoll, 1984a & 1984b; Azouz *et al.* 1994). That synaptically released ACh was acting at mAChRs to inhibition in SFA was confirmed by the inhibition of this response by the mAChR antagonist atropine. The stimulation-evoked inhibition of SFA has been shown to be mediated by an inhibition of a Ca^{++} activated K^+ current I_{AHP} (Lancaster & Adams, 1986; Madison *et al.* 1987). This current mediates the slow afterhyperpolarisation (AHP) which follows a brief depolarization and is the main conductance responsible for the process of SFA (Lancaster & Adams, 1986; see section 1.1.4.1.). As such, a single stimulation of the *s. oriens*, as well as activating a slow depolarizing $EPSP_M$, which is likely to be mediated by an inhibition of $I_{K(LEAK)}$, also inhibits SFA which is likely

to be due to an inhibition of I_{AHP}. Thus, inhibition of I_{AHP} may contribute to the high degree of action potential firing that accompanies activation of an $EPSP_M$ near firing threshold.

3.3.4. OTHER POSTSYNAPTIC mACHR-MEDIATED EFFECTS

Recently, high concentrations of CCh have been shown to modulate action potentials in hippocampal CA1 pyramidal neurones (Figenschou *et al.* 1996). Figenschou and coworkers (1996) demonstrated that CCh increased the duration and, at higher concentrations, reduced the amplitude of action potentials by activation of mAChRs. A broadening of action potentials in response to mAChR stimulation has also been reported to occur in cultured hippocampal neurones (Nakajima *et al.* 1986). Preliminary data presented in this chapter suggests that stimulation of *s. oriens* broadens action potentials evoked in CA1 pyramidal neurones, although no alteration in the amplitude of the action potential was observed. Particularly high concentrations of CCh were required to cause substantial changes reported in the study by Figenschou and coworkers (1996). As such, it is possible that there was no reduction in amplitude in the present study because there was not sufficient activation of mAChRs. This may reflect a limited physiological relevance of this effect. However, small changes in the depolarization produced by an action potential are likely have important consequences in neuronal processing and synaptic transmission, e.g. broadening of action potentials is likely to facilitate the entry of Ca^{++} into neurones. Further investigation using atropine will be required to confirm the observation that physiological activation of mAChRs can broaden action potentials and that this effect is mediated by mAChRs, however, it seems likely that the septo-hippocampal cholinergic input can modulate action potentials evoked in CA1 pyramidal neurones.

3.4. SUMMARY

Stimulation of cholinergic afferents in *s. oriens* with a single stimulus in the presence of ionotropic glutamate and GABA receptor antagonists can evoke reproducible slow $EPSP_M$s in CA1 pyramidal neurones that are associated with an increase in input resistance. In addition, under these conditions, stimulation in *s. oriens*, with a

stimulus intensity sub-threshold for evoking an $EPSP_M$, results in an inhibition of SFA. Preliminary evidence also suggests that stimulation of cholinergic afferents alters the shape of evoked action potentials. Both the $EPSP_M$ and stimulation-induced inhibition of SFA are mediated by mAChRs, although the specific subtype(s) mediating each response remain unclear. Finally, these responses mimic the action of the mAChR agonists such as CCh and AChE inhibitors such as physostigmine.

CHAPTER 4

ADENOSINE RECEPTOR-MEDIATED MODULATION OF MUSCARINIC RECEPTOR-MEDIATED RESPONSES

4.1. INTRODUCTION

In the previous chapter I discussed how stimulation of the septohippocampal input activates a slow excitatory postsynaptic potential ($EPSP_M$) and also reduces SFA, effects which are mediated via mAChR-mediated inhibition of K^+ conductances (Cole & Nicoll, 1983; Cole & Nicoll, 1984a; Madison *et al.* 1987). As with glutamate receptor-mediated effects, such prolonged excitation of CA1 pyramidal neurones, if left unregulated, could be detrimental to these cells and may result in epileptogenesis (Lothman *et al.* 1991; Wasterlain *et al.* 1993). As such, mechanisms that control the magnitude of the mAChR-mediated postsynaptic responses are likely to be of major importance in maintaining the normal functioning of the CNS.

A common mechanism of control of synaptic inputs within the brain is via activation of different receptors to those that mediate the postsynaptic response, e.g. activation of presynaptic heteroreceptors (Thompson *et al.* 1993). As such, the possibility that a neurotransmitter/ neuromodulator other than ACh may restrict the activation of the mAChR-mediated postsynaptic responses was investigated. Adenosine was chosen because this neuromodulator plays an important role in controlling the excitability of neuronal networks by inhibiting other excitatory synaptic inputs, e.g. glutamate (Thompson *et al.* 1992; Thompson *et al.* 1993). Primarily this chapter will deal with how drugs that affect the function of adenosine at the levels of the receptor and its metabolism influence both the $EPSP_M$ and mAChR-mediated reduction in SFA. The possibility that endogenous adenosine in the mammalian brain may tonically modulate cholinergic excitability is also examined.

4.2. RESULTS

4.2.1. EFFECTS OF ADENOSINE RECEPTOR ACTIVATION ON THE $EPSP_M$.

The non-hydrolyzable broad-spectrum adenosine receptor agonist CADO caused a depression of the $EPSP_M$ that was maintained for the period of the agonist application

(n = 19) and was reversed on washout (n = 3; Fig. 4.1). The effect of CADO was concentration-dependent and had an EC_{50} of 0.3 μM (Fig. 4.2). At 0.2 μM CADO caused a substantial depression of the $EPSP_M$ with little or no effect on the postsynaptic membrane potential, input resistance (Fig. 4.3) or SFA in response to a depolarizing current step. In contrast, at concentrations of 0.5 μM and above, the depressant action of CADO on the $EPSP_M$ was invariably accompanied by postsynaptic hyperpolarization (up to 8 mV) and a reduction in input resistance (up to 11 %) (See Fig. 5.1). In routine experiments this hyperpolarization was compensated for by adjusting the current injection into the cell. The maximal effect of CADO on the $EPSP_M$ was 97 % inhibition. However, in one neurone the $EPSP_M$ was unaffected by CADO. Similar results were obtained using adenosine itself. Thus, at 100 μM, adenosine caused a hyperpolarization (Segal, 1982) and inhibited the $EPSP_M$ by 78 ± 10 % (n = 3) (Fig. 4.4*A*).

4.2.2. Pharmacology of the Adenosine Receptor Mediating the Depression of the $EPSP_M$

To elucidate which adenosine receptor subtype was mediating the CADO-induced depression of the $EPSP_M$, the effects of a number of adenosine receptor subtype-specific agonists were investigated. The selective A_1R agonists CCPA (0.1–0.4 μM, n = 7; Fig. 4.4*B*) and R-PIA (1 μM, n = 2; Fig. 4.4*C*) depressed the $EPSP_M$ and caused postsynaptic hyperpolarizations associated with a reduction in input resistance. Thus, for example, at 0.4 μM, CCPA depressed the peak amplitude of the $EPSP_M$ to 34 ± 6 % of that in control medium (n = 4). In contrast, the $A_{2a}R$ agonist CGS 21680, at concentrations up to 1 μM, neither affected significantly the $EPSP_M$ nor the passive membrane properties of the neurones in which it was tested (n = 3; Fig. 4.4*D* & *E*).

The effects of the selective A_1R antagonist DPCPX (0.2 μM) were also tested. DPCPX alone caused a small but variable increase in the size of $EPSP_M$s, with a mean amplitude of 15 mV ("large" $EPSP_M$s), in three out of four neurones studied (Fig 4.5*a*&*c*). As such, the peak amplitude of the $EPSP_M$ in the presence of DPCPX

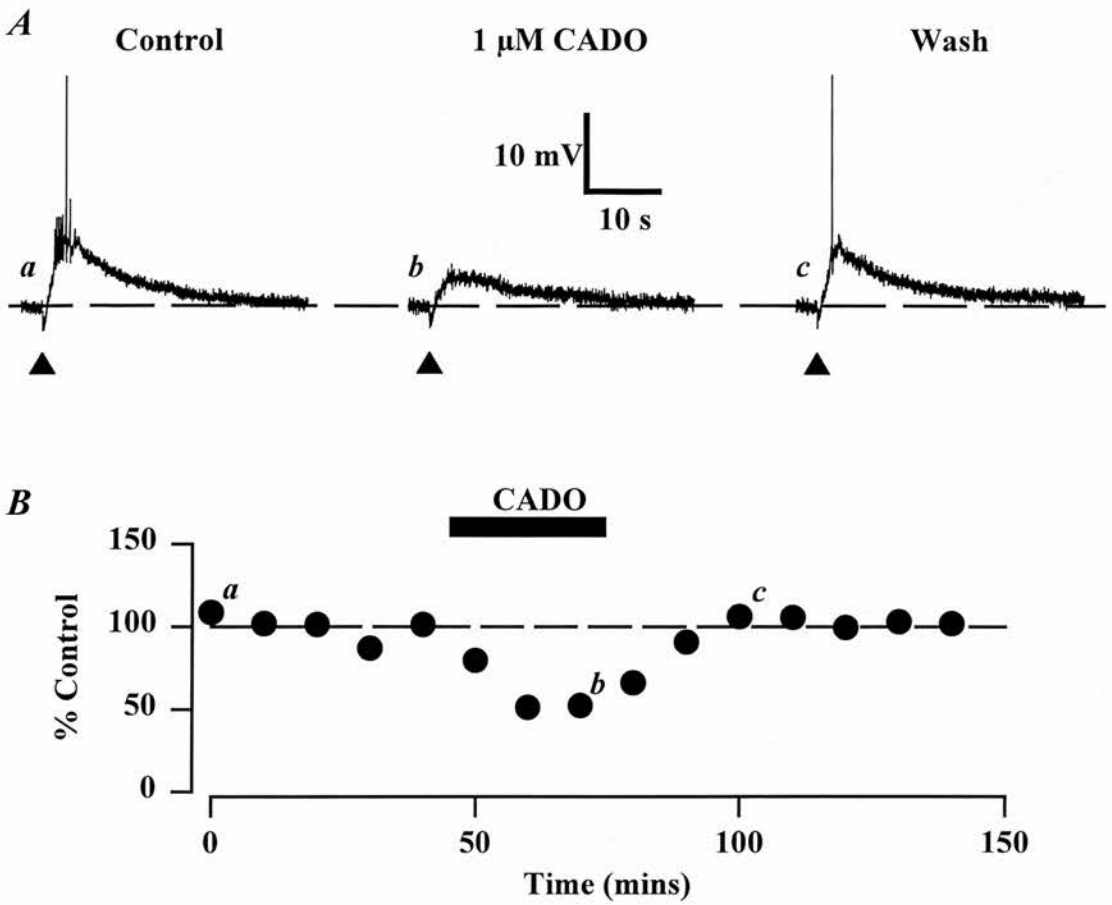

Figure 4.1. The effect of CADO on the $EPSP_M$.

In *A*, synaptic traces are isolated $EPSP_M$s, evoked in response to a single stimulus, recorded in control medium (containing the four ionotropic glutamate and GABA receptor antagonists) (*a*), in the presence of 1 μM CADO (*b*) and following washout (*c*). In this and subsequent figures the point of afferent stimulation is marked by the filled triangles. The graph (*B*) shows a plot of the peak amplitudes of successive $EPSP_M$s normalized to the mean peak amplitude of the five $EPSP_M$s prior to CADO application *versus* time, for a single experiment. CADO was applied for the time indicated by the bar. The points marked *a*, *b* and *c* refer to the synaptic traces illustrated above the graph. Note that CADO reversibly inhibited $EPSP_M$s. The membrane potential of the neurone was –64 mV.

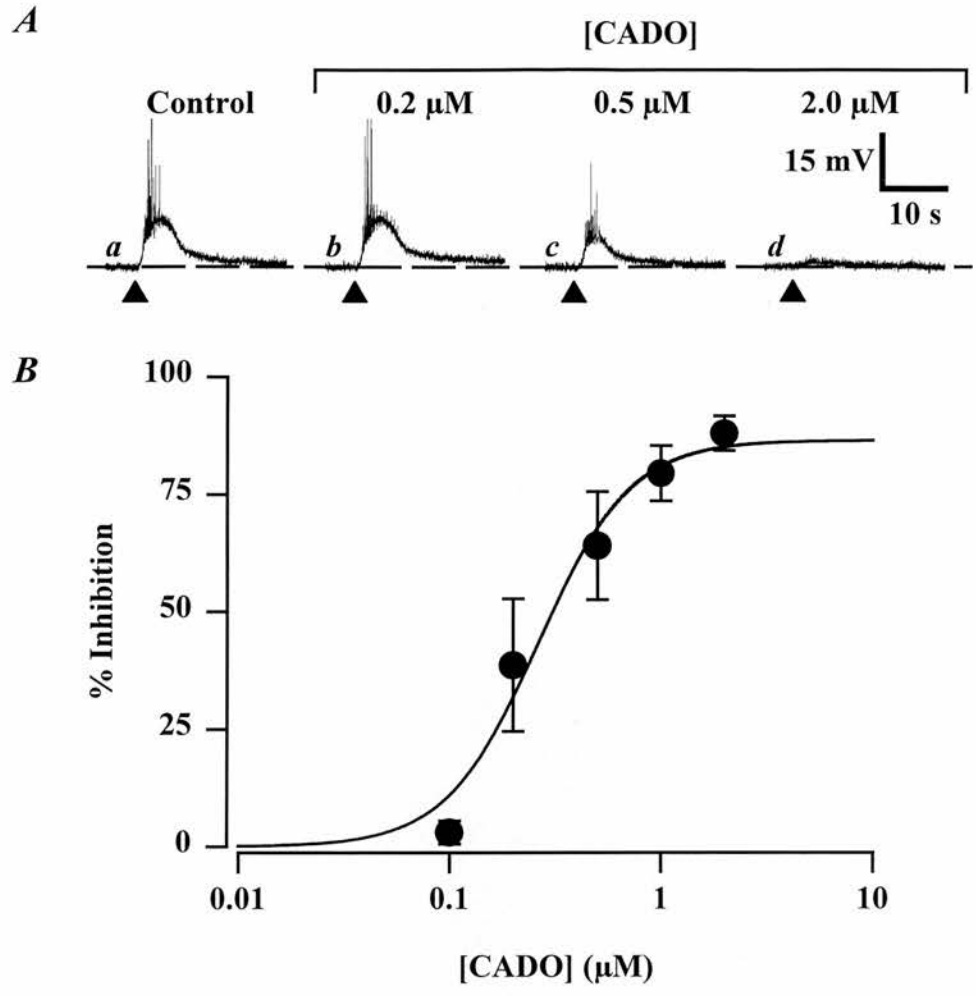

Figure 4.2. The concentration-effect relationship for the effect of CADO on the $EPSP_M$.

A, shows examples of $EPSP_M$s from a single cell, in control medium (*a*) and following the application of CADO at 0.2 μM (*b*), 0.5 μM (*c*) and 2.0 μM (*d*). The membrane potential of the neurone was –67 mV. *B*, plots pooled concentration-effect data for the percentage inhibition of the $EPSP_M$ produced by CADO (0.1–2.0 μM) *versus* concentration of CADO (data from 19 neurones). Percentage inhibition was calculated as the percentage change in the amplitude of $EPSP_M$s in the presence of CADO compared to the mean amplitude of at least three responses recorded in control medium. Each point is the mean value obtained from three to nine separate neurones and the error bars represent the S.E.M. All data (*Y*) were fitted to the logistic expression $Y = M(X^P/[X^P + K^P])$ where *X* is the concentration of CADO, *M* the maximum effect, *K* is the IC_{50} value and the power *P* determines the slope of the sigmoid curve. Note that CADO inhibited $EPSP_M$s in a concentration dependent manner with an IC_{50} of 0.3 μM.

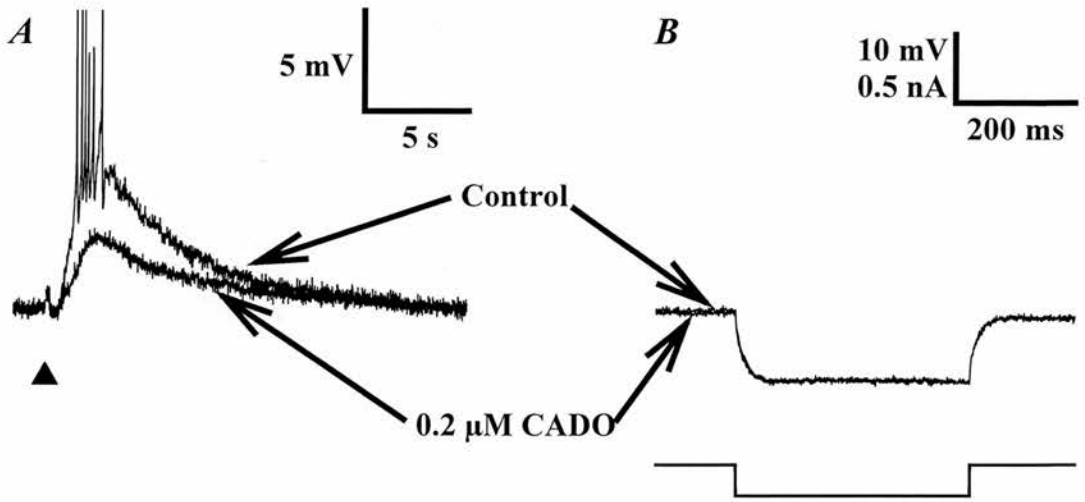

Figure 4.3. A comparison of the effect of 0.2 μM CADO on $EPSP_M$s and on the passive membrane properties of the neurone.

A, shows superimposed traces of $EPSP_M$s in the presence and absence of 0.2 μM CADO. *B*, shows responses to a -0.3 nA current step in the presence and absence of 0.2 μM CADO. As in this and subsequent figures the trace shown below *B* represents the current injected into the cell. Note that 0.2 μM CADO caused a substantial reduction in the $EPSP_M$ without visibly affecting the input resistance of the cell. The membrane potential of the neurone was -64 mV.

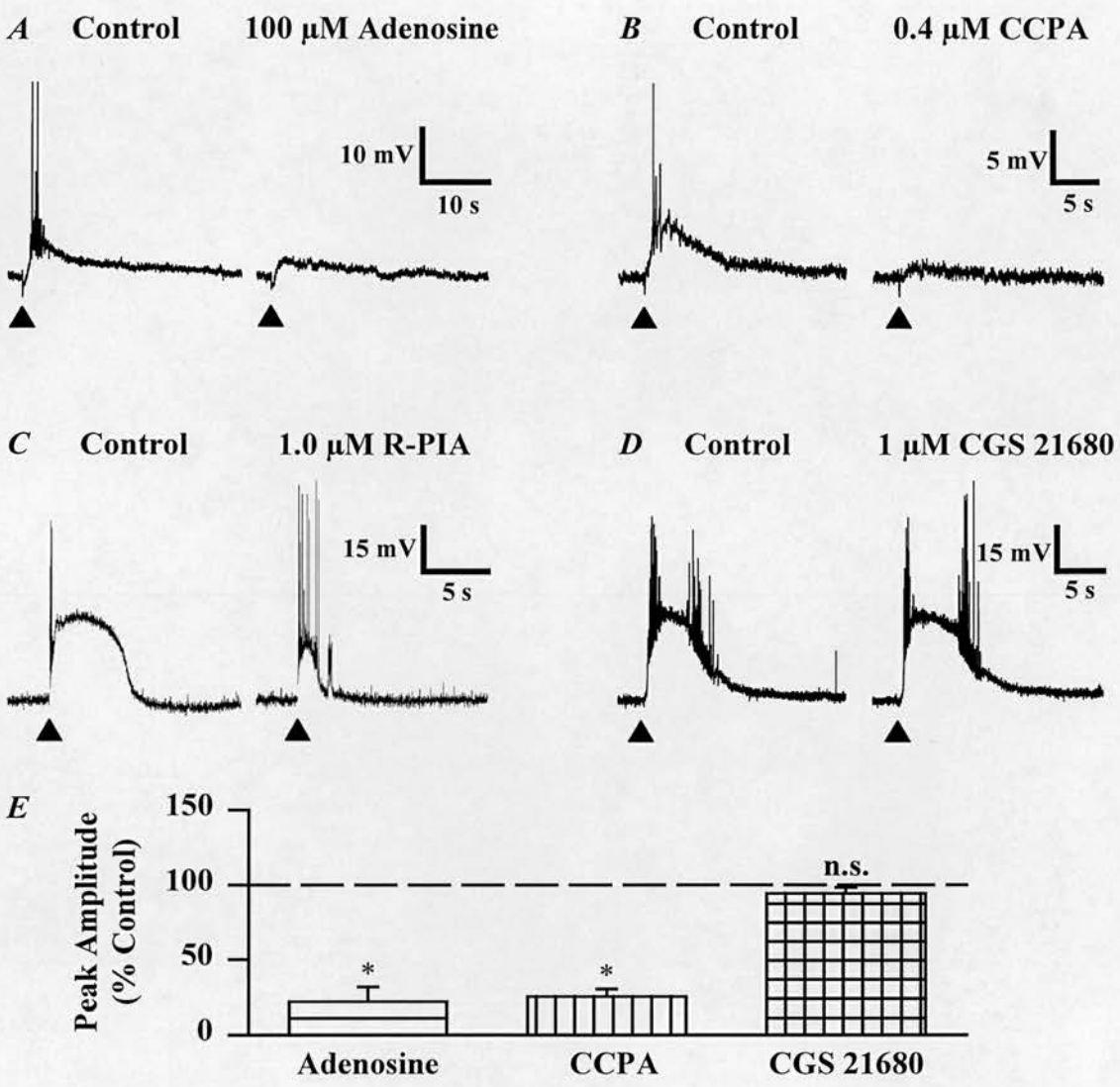

Figure 4.4. Effects of adenosine and subtype selective adenosine receptor agonists on the $EPSP_M$.

In *A–D*, traces illustrate the respective effects of 100 µM adenosine, 0.4 µM CCPA, 1.0 µM R-PIA and 1 µM CGS 21680 on $EPSP_M$s evoked in separate neurones. The membrane potentials of these neurones were -64 mV, -63 mV, -67 mV and -64 mV, respectively. *E*, is a bar graph in which pooled data for the effects of adenosine (100 µM; $n = 3$), CCPA (0.4 µM; $n = 4$) and CGS 21680 (0.5 µM; $n = 3$) on the amplitude of the $EPSP_M$ are expressed as a percentage of the mean value of the control $EPSP_M$s. Note that whilst CGS 21680 had no significant effect adenosine and CCPA significantly inhibited the $EPSP_M$. Data are means ± S.E.M.; * represents $P < 0.05$; n.s., not significant (compared with control).

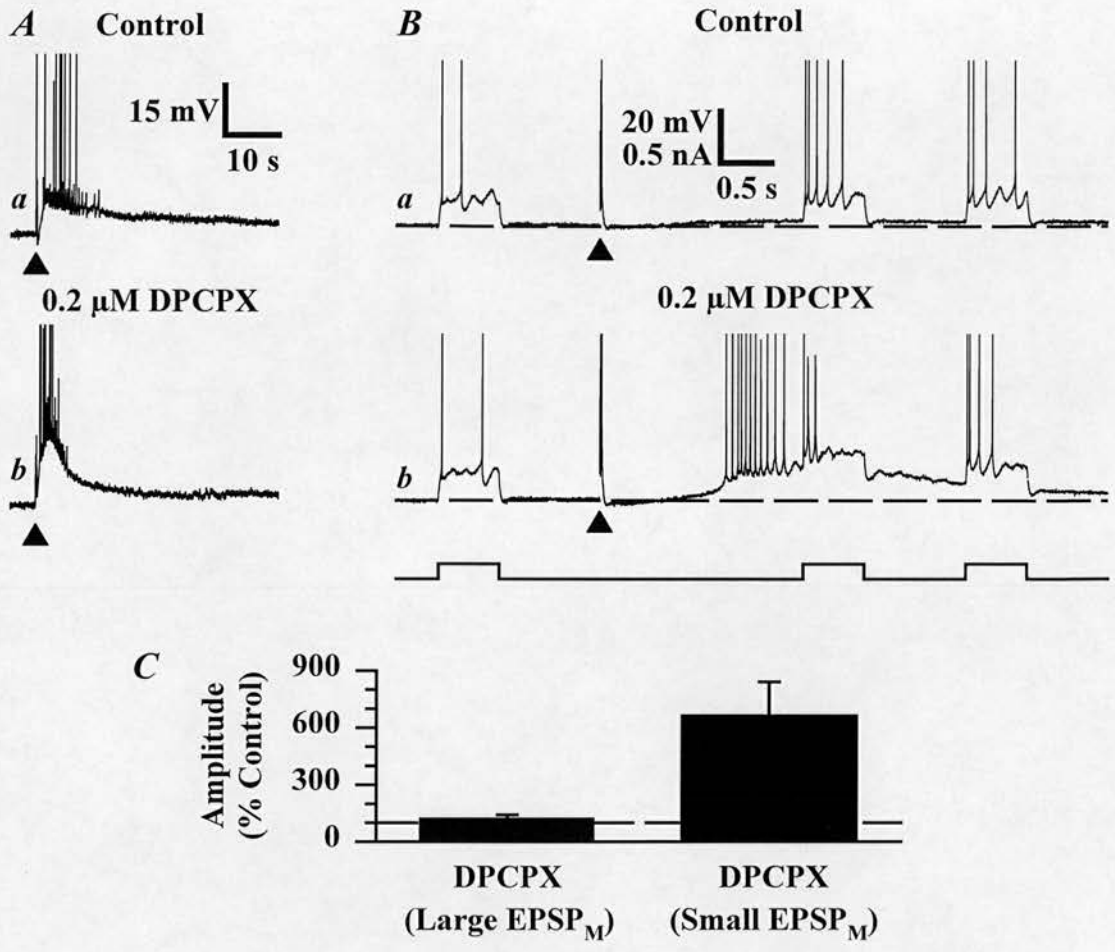

Figure 4.5. The effect of DPCPX on "just suprathreshold" and "large" EPSP$_M$s

In *A*, synaptic traces are isolated EPSP$_M$s, evoked in response to a single stimulus, recorded in control medium (*a*), and in the presence of 0.2 μM DPCPX (*b*). In *B*, traces are continuous records of the membrane potential of a single cell in which a depolarizing current step (+0.2 nA, 600 ms) was delivered 1.0 s prior to, and 2.0 and 3.5 s after pathway stimulation. The stimulation was delivered at an intensity just suprathreshold for activating an EPSP$_M$ in control medium containing only the four ionotropic glutamate and GABA receptor antagonists (*Ba*) and following the addition of 0.2 μM DPCPX (*Bb*). The initial membrane potential of this neurone was –65 mV. Note that in control medium SFA was reduced following pathway stimulation. After addition of DPCPX this intensity of stimulation now additionally activated an EPSP$_M$. *C*, is a bar graph in which pooled data for the effects of DPCPX (0.2 μM) on "large" EPSP$_M$s with a mean peak amplitude of 15 mV ($n = 4$) and "just suprathreshold" EPSP$_M$s with a mean peak amplitude of 1.1 mV ("Small"; $n = 3$). Values were calculated as described in Figure 4.4*E*. Note that DPCPX had a relatively small effect on large EPSP$_M$s but greatly increased the amplitude of small EPSP$_M$s.

was 122 ± 19 % that of control (n = 4). In addition, in the presence of DPCPX, a stimulus that was previously sufficient to reduce SFA but just supra-threshold for evoking an $EPSP_M$ ("small" $EPSP_M$) subsequently evoked an $EPSP_M$ that had a peak amplitude of 8.3 ± 3.9 mV (n = 3; Fig. 4.5*b*&*c*).

Next the ability of DPCPX to antagonize the depressant effects of CADO and adenosine was examined. DPCPX (0.2 μM) fully reversed, or prevented, the inhibition of the $EPSP_M$ by CADO (1 μM; n = 6; Fig. 4.6*A*) and by adenosine (100 μM; n = 3; 4.6*B*). DPCPX made the $EPSP_M$ larger than control responses in a number of neurones and also inhibited the postsynaptic hyperpolarization and decrease in input resistance evoked by CADO (See Fig. 5.1) and adenosine (data not shown).

4.2.3. Effects of CADO on the Reduction in Spike Frequency Adaptation Evoked by Endogenous Acetylcholine

As described in section 3.2.5, stimulation that was sub-threshold for activating the $EPSP_M$ caused a reduction in SFA in response to a depolarizing current step delivered 2 s after pathway stimulation (n = 12; Fig. 4.7*A*). This reduction in SFA was inhibited by CADO (Fig. 4.7*B*) in a concentration-dependent manner (1–5 μM; n = 3; Fig. 4.8) and was re-instated by subsequent application of DPCPX (0.2 μM; n = 3; Fig. 4.7*C* & 4.8*E*). In two neurones, in the combined presence of CADO and DPCPX a small $EPSP_M$ was evoked by the previously sub-threshold stimulus (Fig. 4.7*C*). This $EPSP_M$, along with the reduction in SFA, was abolished by subsequent application of atropine (1 μM; Fig. 4.8*F*).

4.2.4. Effects of an Adenosine Kinase Inhibitor on the $EPSP_M$

The increase in the $EPSP_M$ that was caused by DPCPX alone suggested that endogenous adenosine was capable of tonically activating the A_1Rs that subsequently inhibit the $EPSP_M$. Therefore, the possibility that inhibition of the breakdown of adenosine might potentiate this putative tonic effect was tested. This was achieved using the selective adenosine uptake inhibitor dipyridamole (DPY) and the adenosine kinase inhibitor 5-iodotubercidin (5-IT), which raise extracellular adenosine levels by

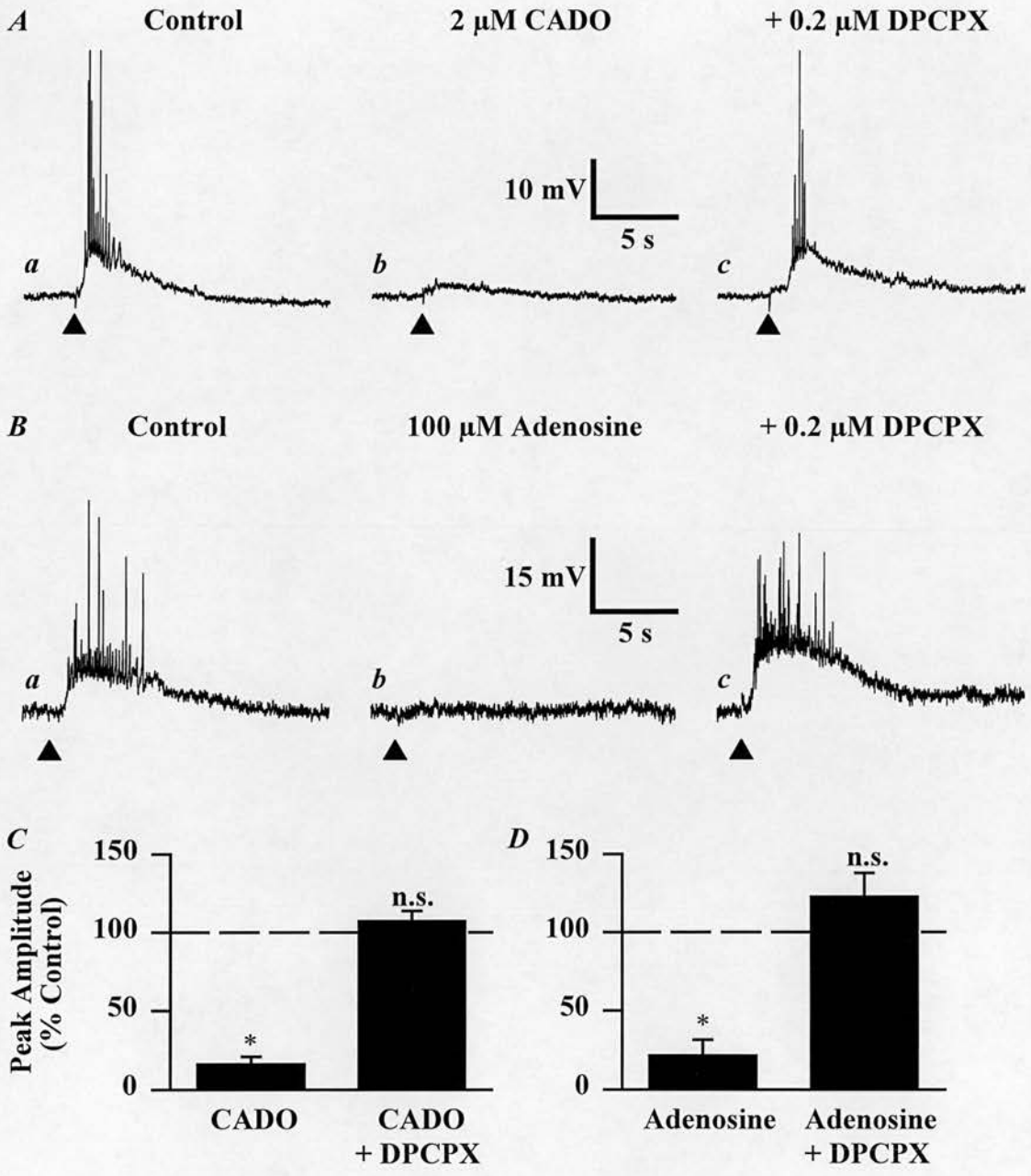

Figure 4.6. The effect of DPCPX on the adenosine and CADO-induced depression of $EPSP_M$s.

In *A* and *B*, synaptic traces are $EPSP_M$s recorded in control medium (*Aa* & *Ba*), in the presence of 1 μM CADO (*Ab*) or 100 μM adenosine (*Bb*) and in the additional presence of 0.2 μM DPCPX (*Ac* & *Bc*). The membrane potential of both cells was -64 mV. The bar graphs illustrate pooled data for the effects of 1 μM CADO ($n = 8$) and 1 μM CADO + 0.2 μM DPCPX ($n = 4$) (C) and the effects of 100 μM adenosine ($n = 3$) and 100 μM adenosine + 0.2 μM DPCPX ($n = 3$) (D) on the $EPSP_M$. Each value was calculated as described for Fig. 4.4*E*. Note that both CADO and adenosine significantly depressed the $EPSP_M$ and responses in the presence of either CADO or adenosine and DPCPX were not significantly different from control responses. Data are means ± S.E.M.; * represents $P < 0.05$; n.s., not significant (compared with control).

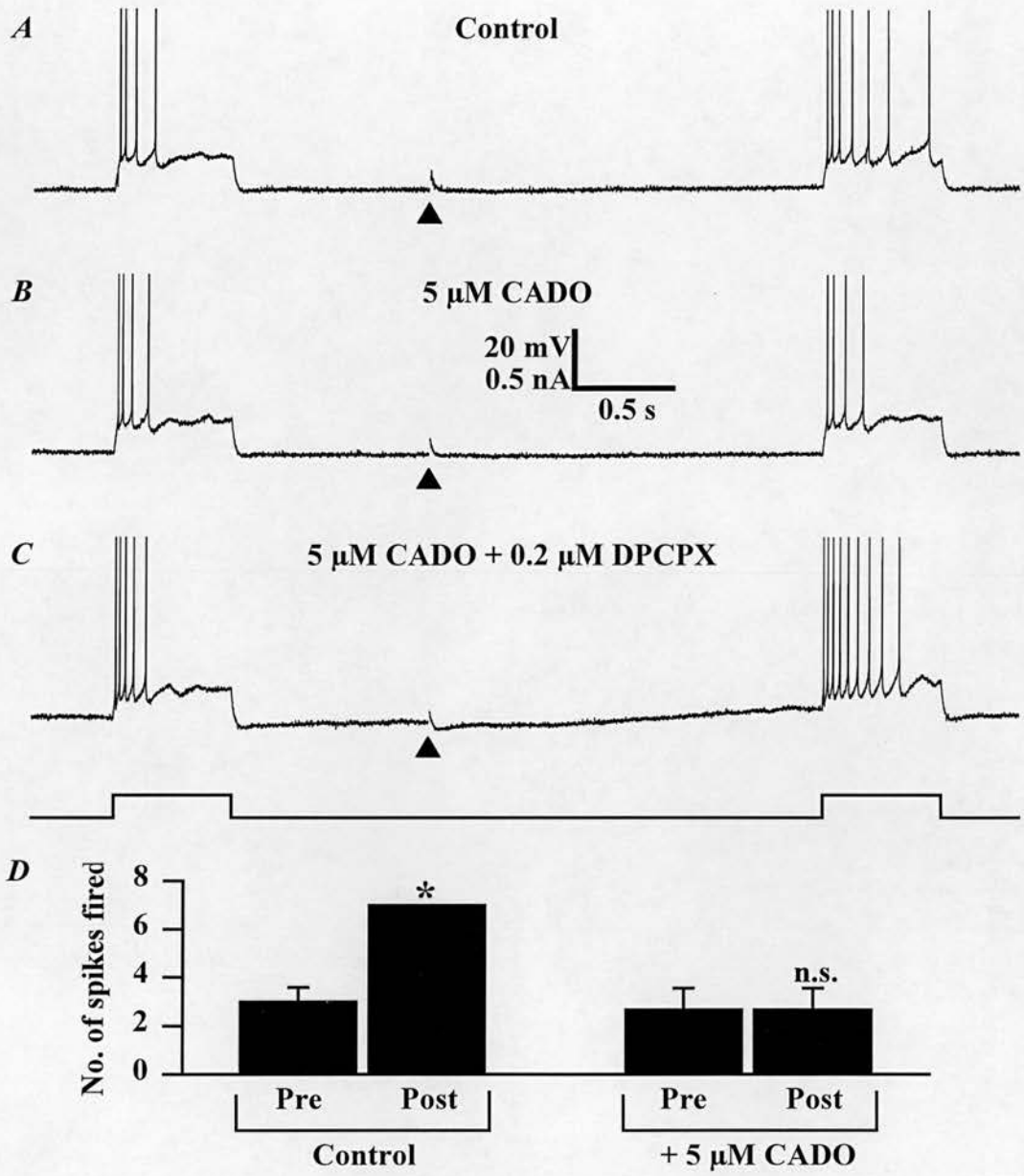

Figure 4.7. The effect of DPCPX on the CADO-induced inhibition of stimulation-evoked reduction in spike frequency adaptation.

In *A–C*, traces are generated as described in Figure 4.5*B*. The responses were obtained in medium containing the four ionotropic glutamate and GABA receptor antagonists (*A*), in the additional presence of 5 μM CADO (*B*) and in the additional combined presence of 5 μM CADO and 0.2 μM DPCPX (*C*). Note that in control medium SFA was reduced following pathway stimulation, and that this reduction was abolished by CADO in a DPCPX-sensitive manner. *D*, shows pooled data for the number of action potentials fired during a depolarizing step 1.0 s before (Pre) and 2.0 s after (Post) a sub-threshold stimulus in the absence and presence of 5 μM CADO ($n = 3$). If two current steps were delivered without intervening sub-threshold stimulation the level of SFA evoked by the first step was not significantly different from that evoked by the second step (See Fig. 3.10). In addition, note that in the presence of CADO + DPCPX previously sub-threshold pathway stimulation now evoked a small $EPSP_M$ that was abolished by the subsequent addition of atropine (not shown). The initial membrane potential of the neurone was -66 mV.

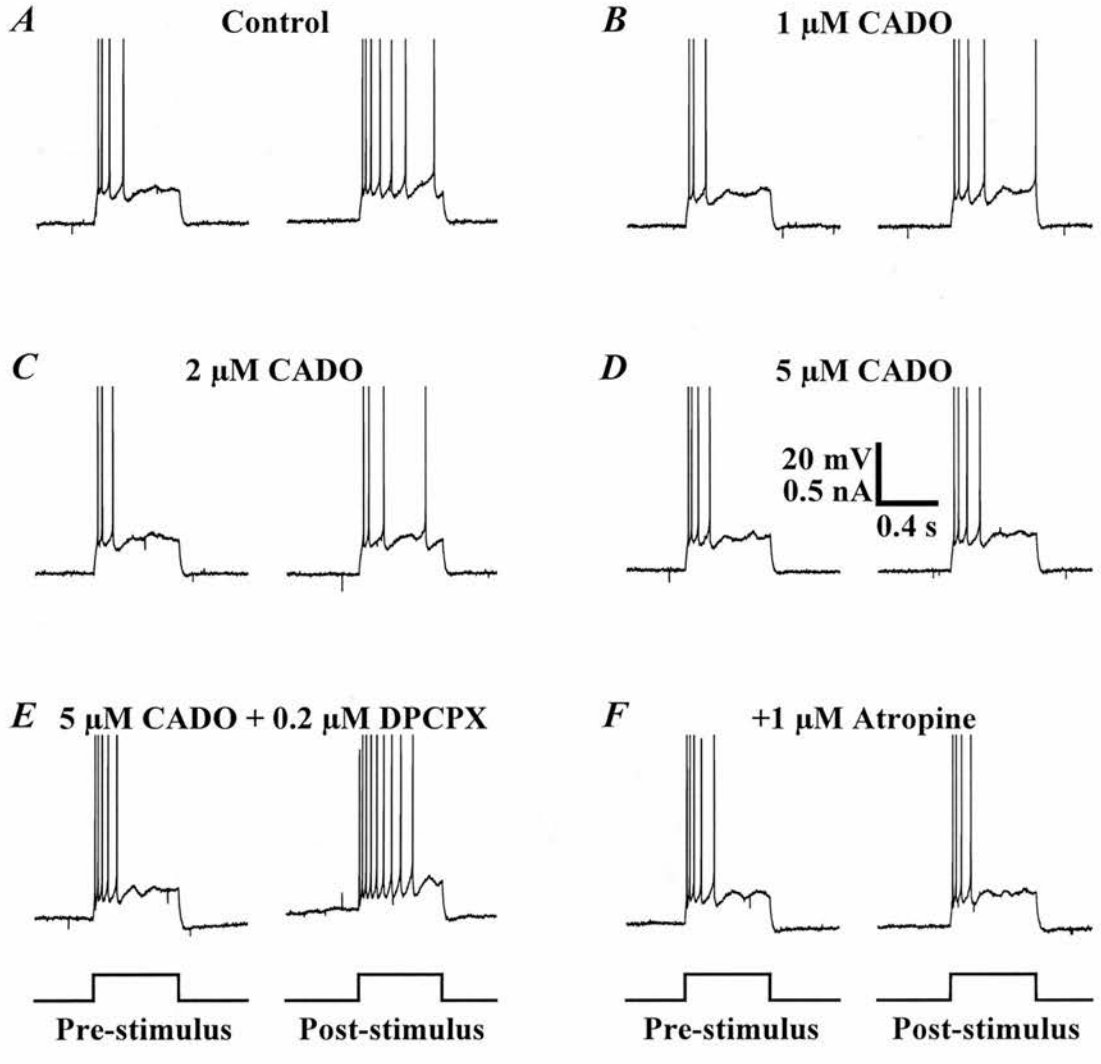

Figure 4.8. The concentration-dependent effect of CADO on stimulation evoked inhibition of spike frequency adaptation.

In *A–F*, traces are generated as described in Fig. 4.5 however the point of pathway stimulation has been omitted by a 2 s break, marked by the gap between steps, which has been added for clarity. The responses were obtained in medium containing the four ionotropic glutamate and GABA receptor antagonists (*A*), in the additional presence of 1 μM (*B*), 2 μM (*C*), 5 μM (*D*) CADO, in the combined presence of 5 μM CADO and 0.2 μM DPCPX (*E*) and following subsequent addition of 1 μM atropine (*F*). Note that in control medium SFA was reduced following pathway stimulation, that this reduction was inhibited in a concentration dependent manner by CADO. DPCPX reversed this inhibition such that the neurone subsequently evoked an inhibition of SFA and a depolarization, which were completely inhibited by atropine. The initial membrane potential of this neurone was -64 mV.

preventing uptake of adenosine and inhibiting the conversion of adenosine into adenosine 5'-monophosphate (AMP), respectively (Jackisch *et al.* 1984; Ohkubo *et al.* 1991; Pak *et al.* 1994). Both DPY (10 μM) and 5-IT (10 μM) caused a postsynaptic hyperpolarization (2–8 mV), a decrease in input resistance (5–15 %) and a depression of the $EPSP_M$ by 62 ± 7 % and 74 ± 10 % respectively ($n = 4$ & $n = 5$; Fig 4.9*A–C*). However, in three neurones in which 5-IT was tested, subsequent application of DPCPX (0.2 μM) completely reversed these effects (Fig. 4.9*A*). In contrast, DPCPX (0.2 μM) had little or no effect on depression of $EPSP_M$s by DPY (10 μM; $n = 3$; data not shown).

4.3. DISCUSSION

4.3.1. ADENOSINE RECEPTOR ACTIVATION INHIBITS CHOLINERGIC SYNAPTIC TRANSMISSION

In this chapter, compelling and original evidence has been presented showing that adenosine receptor activation depresses $EPSP_M$s and, in addition, prevents the reduction in SFA mediated by synaptic activation of mAChRs. Until now there has been only limited evidence of pharmacological modulation of cholinergic synaptic transmission. Most studies have focused on the modulation of release of ACh evoked by stimulation using high concentrations of K^+ and strong electrical stimuli (Harms *et al.* 1979; Cunha *et al.* 1994; Kirk & Richardson, 1994). Although this technique provides a good model of release systems, it may have limited physiological significance due to the artificially high levels of stimulation used to release neurotransmitters. Nevertheless, such neurochemical release studies have demonstrated that evoked release of ACh can be modulated by adenosine receptor activation at both peripheral and central synapses (Vizi & Knoll, 1976; Cunha *et al.* 1994). Moreover, adenosine has been shown to modulate the release of glutamate as well as glutamate receptor-mediated synaptic responses in the hippocampus (de Mendonça & Ribeiro, 1993; Thompson *et al.* 1992).

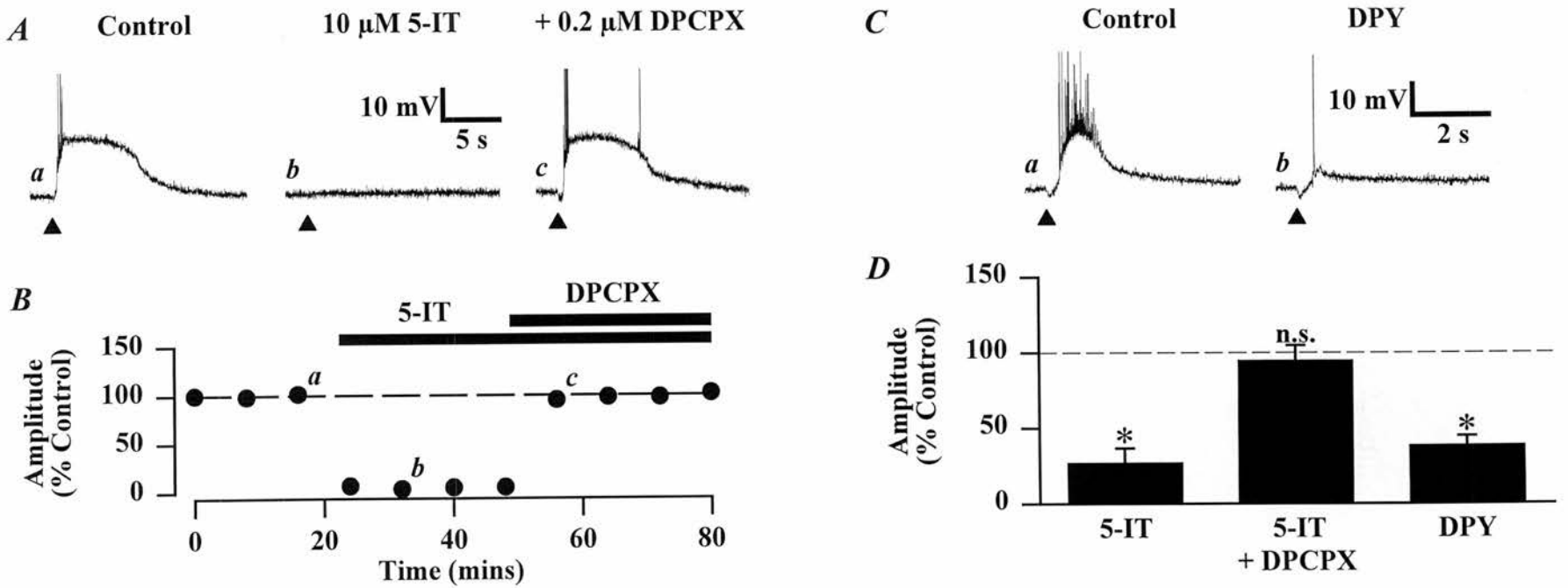

Figure 4.9. The effects of inhibitors of adenosine kinase and of adenosine uptake on the $EPSP_M$.

In *A*, synaptic traces are representative $EPSP_M$s recorded in control (*a*), in the presence of 5-iodotubercidin (5-IT, 10 μM) (*b*) and in the presence of 5-IT (10 μM) + DPCPX (0.2 μM) (*c*). The membrane potential of this neurone was -66 mV. In *B*, The graph is similar to that illustrated in Fig. 4.1*B* and plots the peak amplitude of successive $EPSP_M$s, for a single experiment, *versus* time to illustrate the depressant effect of 5-IT on the $EPSP_M$ and its reversal by DPCPX. The bars above the graph indicate the duration for which the two drugs were applied. In *C*, synaptic traces are representative $EPSP_M$s recorded in control (*a*) and in the presence of dipyridamole (DPY, 10 μM) (*b*). The membrane potential of this neurone was -64 mV. *D*, is a bar graph illustrating pooled data for the effects of 10 μM 5-IT ($n = 4$), 10 μM 5-IT + 0.2 μM DPCPX ($n = 3$) and 10 μM DPY ($n = 5$) on the $EPSP_M$. The values plotted were calculated as described in figure 4.4*E*. Note that both 5-IT and DPY significantly depressed the $EPSP_M$ ($P < 0.05$) and that responses in the combined presence of 5-IT and DPCPX were not significantly different from controls ($P > 0.05$).

Functional electrophysiological evidence of modulation of cholinergic synaptic transmission has, however, been limited. In one study, the neuropeptide galanin depressed slow cholinergic EPSPs, evoked in the presence of physostigmine, by approximately 60 % via a presynaptic mechanism in the ventral hippocampus (Dutar *et al.* 1989). In the present study, $EPSP_M$s were inhibited by adenosine receptor activation. These $EPSP_M$s were evoked in the absence of a cholinesterase inhibitor and, as such, these results provide the first functional evidence of a modulation of cholinergic synaptic transmission in the CNS where the metabolism of ACh is unimpaired. Furthermore, these results provide evidence of a modulation of cholinergic effects on the firing properties of the postsynaptic neurone, namely SFA, an effect which may be more likely to mimic the modulatory effects of ACh *in vivo*.

Unlike the study by Dutar and coworkers (1989), no distinction was made between dorsal and ventral hippocampus in the present study, therefore these depressant actions of adenosine are likely to operate throughout the entire CA1 region of the hippocampus. Interestingly, a differential distribution of A_1Rs has been noted between dorsal and ventral hippocampus, which has been correlated to the differential regulation of glutamatergic synaptic responses by adenosine (Lee *et al.* 1983). More careful consideration of the effects of adenosine on cholinergic responses evoked in such distinct regions of the hippocampus may provide similar results. This may also provide some explanation of the large variability of the effect of CADO and adenosine on the amplitude of $EPSP_M$s. Alternatively, this may reflect variation in the concentration of endogenous adenosine in the slice at the time of recording (Dunwiddie & Diao, 1994). A much larger concentration of adenosine than that of CADO was required to inhibit $EPSP_M$s. This is in agreement with previous studies investigating other synaptic responses and may be due, in part, to

i) the relative stability of CADO to breakdown by ectoenzymes

ii) uptake of adenosine and,

iii) the lower potency of adenosine at A_1Rs (Dalziel & Westfall, 1994).

$EPSP_M$s were not significantly affected by CGS 21680, suggesting that A_{2a}Rs do not mediate the depression of $EPSP_M$s by CADO. Coupled with the high potency of CADO, which is relatively more potent at A_1Rs, this indirectly suggests that A_1Rs mediate this depression. This was confirmed by the depression of $EPSP_M$s by the selective adenosine A_1 receptor agonists CCPA and R-PIA and by the inhibition of these effects by the selective adenosine A_1 receptor antagonist DPCPX. Although DPCPX is relatively selective for A_1Rs it also has very low potency at A_{2b}Rs. However, the potent effect of DPCPX in conjunction with the effect of the selective A_1R agonist CCPA tend to exclude the involvement of an A_{2b}R in the response to CADO. The action of DPCPX also excludes possible effects at A_3Rs, since DPCPX is not a potent antagonist at rodent A_3Rs (Zhou *et al.* 1992). In addition, binding studies have revealed only low levels of A_3R binding in the mouse hippocampus (Jacobson *et al.* 1993).

This system of regulation of $EPSP_M$s is analogous to that at glutamate synapses in the CA1 region, where A_1Rs inhibit both AMPA and NMDA receptor-mediated synaptic transmission (de Mendonça & Ribeiro, 1993; Thompson *et al.* 1992). The IC_{50} value for the CADO-induced depression of the $EPSP_M$ (0.3 μM) is slightly less than that observed for inhibition of AMPA receptor-mediated EPSPs but is similar to that for the inhibition of NMDA receptor-mediated EPSPs by CADO (de Mendonça & Ribeiro, 1993; Dunwiddie & Diao, 1994). It is possible that this reflects the greater need for control of cholinergic and NMDA receptor-mediated synaptic inputs because of their much greater influence on postsynaptic excitability.

The lack of a significant effect of CGS 21680 is not surprising since autoradiographic studies using [^{3}H] CGS 21680 have found little A_{2a}R binding in area CA1 of the hippocampus (Jarvis & Williams, 1989). However, A_{2a}Rs may regulate cholinergic synaptic transmission in other areas of the hippocampus, since activation of this receptor has been shown to potentiate release of ACh in the CA3 and dentate gyrus regions, but not the CA1 region (Cunha *et al.* 1994). Despite this, CGS 21680 has been shown to inhibit both field recorded glutamate receptor-mediated EPSPs and

low Ca^{++} bursting (a putative postsynaptic effect) in the hippocampal CA1 region (Lupica *et al.* 1990). However, these effects have been attributed to a lack of selectivity of CGS 21680 at the concentrations used (10–100 μM), which were much higher than those used in the present study (0.5–1.0 μM). As such, the previously reported effects were thought to be mediated by a non-selective action of CGS 21680 at A_1Rs (Lupica *et al.* 1990).

Inhibition of stimulation-evoked reduction of SFA by CADO was also mediated by DPCPX-sensitive receptors, suggesting that the receptors mediating this effect are A_1Rs. However, other than their similar pharmacology, there is no direct evidence to suggest that these receptors are from the same population as those responsible for inhibiting $EPSP_M$s. Indeed a higher concentration of CADO (5 μM) than that required to inhibit the $EPSP_M$ (1 μM) was required to completely inhibit this response. This may, however, reflect the need to inhibit more completely cholinergic transmission, as a lower level of mAChR activation is required to mediate inhibition of SFA than to evoke an $EPSP_M$ (Madison *et al.* 1987). The sensitivity of the responses used in this study may be limited, as a substantial inhibition of I_{AHP} may be required to significantly inhibit SFA. These observations may be further complicated by a possible discrepancy between the receptor-effector coupling for $I_{K(LEAK)}$ and I_{AHP}. Moreover, it is possible that different populations of mAChRs mediate inhibition of $I_{K(LEAK)}$ and I_{AHP}. In this respect mAChRs may differ in their location (synaptic or extrasynaptic) or in their pharmacological properties. Until these possibilities are fully investigated, the significance of the differential effects of CADO in mAChR - mediated inhibition of $I_{K(LEAK)}$ and I_{AHP} will remain uncertain.

4.3.3. Mechanism of Action of Adenosine

Data presented in this chapter does not provide any direct evidence of the mechanism of adenosine A_1 receptor-mediated depression of cholinergic synaptic responses. In area CA1 of the hippocampus, adenosine has been shown to activate K^+ conductances postsynaptically that lead, for example, to a hyperpolarization and decrease in input resistance (Thompson *et al.* 1992). Activation of postsynaptic A_1Rs has also been shown to enhance the sAHP and SFA by affecting I_{AHP} (Haas &

Greene, 1984). This may affect subsequent inhibition of I_{AHP} by mAChR activation. Presynaptically, adenosine could be inhibiting release of ACh by affecting K^+ or Ca^{++} channels or alternatively by directly affecting the release machinery (see section 1.2.4.3.).

There is, however, some indirect evidence as to the locus of the A_1R-mediated effects. In this respect, a presynaptic locus may be tentatively suggested because activation of A_1Rs inhibits both $EPSP_M$s and mAChR-mediated inhibition of SFA. These effects are thought to be mediated by two separate mechanisms i.e. inhibition of $I_{K(LEAK)}$ and I_{AHP} (Madison *et al.* 1987). Therefore the most simplistic explanation is that adenosine A_1 receptor activation is acting at a point upstream of the postsynaptic mAChR-mediated signal transduction mechanisms i.e. inhibition of transmitter release. In addition, a presynaptic locus is suggested as A_1R activation can lead to a depression of the $EPSP_M$ with little or no observable postsynaptic electrophysiological effects. This, however, does not take into account possible effects on the postsynaptic biochemistry which do not lead to observable electrophysiological changes e.g. effects on G-protein-receptor interactions. Numerous studies citing the inhibitory effect of adenosine receptor activation on the turnover or release of ACh in the hippocampus, striatum and cortex, both *in vivo* and *in vitro*, support a presynaptic locus for these effects (e.g. Cunha *et al.* 1994; Harms *et al.* 1979; Murray *et al.* 1982; Haubrich *et al.* 1981). This issue will be addressed in the context of further experiments presented in chapter 5.

4.3.4. Endogenous Adenosine Activates Adenosine Receptors

As at glutamatergic synapses (Greene *et al.* 1985; Bauman *et al.* 1992), a degree of "adenosinergic" inhibition at cholinergic synapses may be tonically active in the hippocampal slice, as suggested by the enhancement of the $EPSP_M$ by DPCPX. This putative "purinergic tone" appears to be greatly enhanced when the uptake or metabolism of adenosine is impaired by DPY or 5-IT respectively. 5-IT and DPY have also been shown to facilitate the action of endogenous adenosine on glutamatergic synaptic transmission (Mitchell *et al.* 1993; Pak *et al.* 1994; Diao & Dunwiddie, 1996). While the reversal of the effect of 5-IT by DPCPX suggests an

increased action of adenosine at A_1Rs, the lack of effect of DPCPX on the action of DPY would suggest that DPY is not acting via activation of A_1Rs by endogenous adenosine. This is surprising, as DPY is believed to be a selective inhibitor of adenosine uptake at the concentration used (10 μM). In this respect, one study, investigating the release of [^{3}H]ACh from rabbit hippocampal slices, DPY decreased the evoked release of ACh and 8-phenyltheophylline reversed this effect, suggesting a selective effect of DPY on adenosine uptake (Jackisch *et al.* 1984). Nevertheless, the inhibitory action of DPY on $EPSP_M$s is in agreement with neurochemical release studies where DPY was shown to inhibit release of ACh from rabbit hippocampal slices (Jackisch *et al.* 1984) and rat cortical slices (Pedata *et al.* 1983a).

The distribution of A_1Rs in the CNS is generally closely associated with the distribution of adenosine uptake sites. In the hippocampus however, adenosine uptake sites, labeled by [^{3}H]NBTI, are not as closely associated with A_1Rs as in other areas (e.g. striatum) (Glass *et al.* 1996). This may indicate a less important role for adenosine uptake in the hippocampus. Possibly this reflects the need to control the relatively high excitability generated in this region by activation of NMDA receptors and mAChRs, the hippocampus being the region with the highest density of both these receptors in the rat brain (Monaghan & Cotman, 1985; Maragos *et al.* 1988).

The source of the putative endogenous adenosine tonus is, however, unclear. One possibility is that it is due, at least in part, to non-specific accumulation of adenosine due to cell damage resulting from slice preparation (Thompson *et al.* 1992). Another possibility is that adenosine is released following afferent stimulation (Mitchell *et al.* 1993) from, for example, GABAergic interneurones (Manzoni *et al.* 1994). Whatever the case, there is considerable evidence that the level of extracellular adenosine in slices is comparable to that in the CNS *in vivo* (Zetterström *et al.* 1982; Dunwiddie & Diao, 1994; Fredholm *et al.* 1984). As such, it is likely that in the intact animal cholinergic, like glutamatergic, synaptic inputs in the hippocampus are tonically, and perhaps subtly, inhibited by circulating levels of adenosine.

4.4. SUMMARY

Adenosine receptor activation inhibits mAChR-mediated synaptic responses in the CA1 region of the rat hippocampus. The agonist and antagonist profile of this receptor shows that it closely resembles the adenosine A_1 receptor, which also inhibits glutamatergic synaptic transmission in this region. Data presented in this chapter also suggest that there is a tonic activation of these receptors *in vitro* and this provides a potentially important mechanism for the control of neuronal excitability in CA1 pyramidal neurones *in vivo*.

CHAPTER 5

PRE- AND POST-SYNAPTIC ADENOSINE RECEPTORS: LOCUS AND MECHANISM OF ADENOSINE RECEPTOR-MEDIATED EFFECTS ON CHOLINERGIC SYNAPTIC TRANSMISSION

5.1. INTRODUCTION

As previously discussed, adenosine receptor activation has a variety of effects in the mammalian hippocampus and these are, through a variety of mechanisms, predominantly inhibitory (see section 1.2). In the previous chapter, it was shown that A_1Rs have an inhibitory modulatory role at cholinergic synapses in the rat hippocampus. However, it is unclear whether this effect is mediated pre- or post-synaptically. In this respect, there is much experimental evidence to suggest that A_1Rs are located both pre- and postsynaptically in the CNS (Proctor & Dunwiddie, 1987; Thompson *et al.* 1992; Swanson *et al.* 1995). As such, A_1R-mediated modulation of cholinergic synaptic transmission could occur presynaptically, e.g. by an inhibition of transmitter release, or postsynaptically by interfering with the transduction of mAChR-mediated responses e.g. by affecting G-protein function. At glutamatergic synapses in the hippocampus, adenosine receptor activation modulates synaptic transmission via a presynaptic receptor (Dunwiddie & Hoffer, 1980; Dunwiddie & Haas, 1985; Proctor & Dunwiddie, 1987). However, the precise mechanism of action of adenosine, i.e. whether or not A_1Rs inhibit presynaptic Ca^{++} currents, in this system remains controversial (Silinsky, 1986; Fredholm & Dunwiddie, 1988; Dunwiddie, 1990; Wu & Saggau, 1997).

This chapter deals directly with the issue of determining either a pre- or post-synaptic locus for the A_1Rs that modulate cholinergic synaptic responses in area CA1 of the rat hippocampus. Particular emphasis is placed on the comparison of the effect of A_1R activation on postsynaptic responses to mAChR agonists with that on responses due to synaptic activation of mAChRs. Having dealt with the locus of the A_1Rs which mediate this response, some preliminary work is presented relating to the transduction mechanism(s) involved in the A_1R-mediated modulation of mAChR-mediated synaptic responses.

5.2. RESULTS

Having identified the A_1R-mediated depression of cholinergic synaptic responses in chapter 4, the next group of experiments addressed whether pre- or post-synaptic A_1Rs mediate these effects. Initially, the concentration-response relationships for known pre- and post-synaptic A_1R-mediated effects were compared with that for A_1R-mediated depression of $EPSP_M$s.

5.2.1. THE PRE- AND POSTSYNAPTIC EFFECTS OF ADENOSINE RECEPTOR ACTIVATION

5.2.1.1. The effects of CADO on the passive membrane properties of the neurone

Activation of postsynaptic adenosine receptors causes membrane hyperpolarization and reduction in input resistance in hippocampal pyramidal neurones (Greene & Haas, 1985; Gerber *et al.* 1989). CADO (0.2–50 μM) caused a hyperpolarization with an EC_{50} of 3.0 μM and a reduction in input resistance with an EC_{50} of 3.2 μM (Fig. 5.1). Both the reduction in input resistance and the hyperpolarization were reversed by DPCPX (0.2 μM; Fig. 5.1*A*).

5.2.1.2. The effects of CADO on ionotropic glutamate-receptor mediated responses

There is a considerable body of evidence indicating that activation of A_1Rs presynaptically causes a depression of glutamate-mediated EPSPs (Lupica *et al.* 1992). Initially, experiments were performed investigating the effects of CADO on field recorded EPSPs and subsequently on intracellularly recorded, isolated AMPA receptor-mediated EPSPs ($EPSP_A$s; Fig. 5.2). Intracellular experiments were used so that postsynaptic membrane potential changes, that would affect field recorded EPSPs, could be compensated for using DC current injection. Despite this, CADO (0.2–5.0 μM) inhibited both field recorded EPSPs and $EPSP_A$s with EC_{50}s of 0.8 μM (Fig. 5.2*C*). These effects of CADO were reversed by DPCPX (0.2 μM) and $EPSP_A$s were inhibited by the selective AMPA receptor antagonist NBQX (Fig. 5.2 *A* & *B*; 2 μM; $n = 4$).

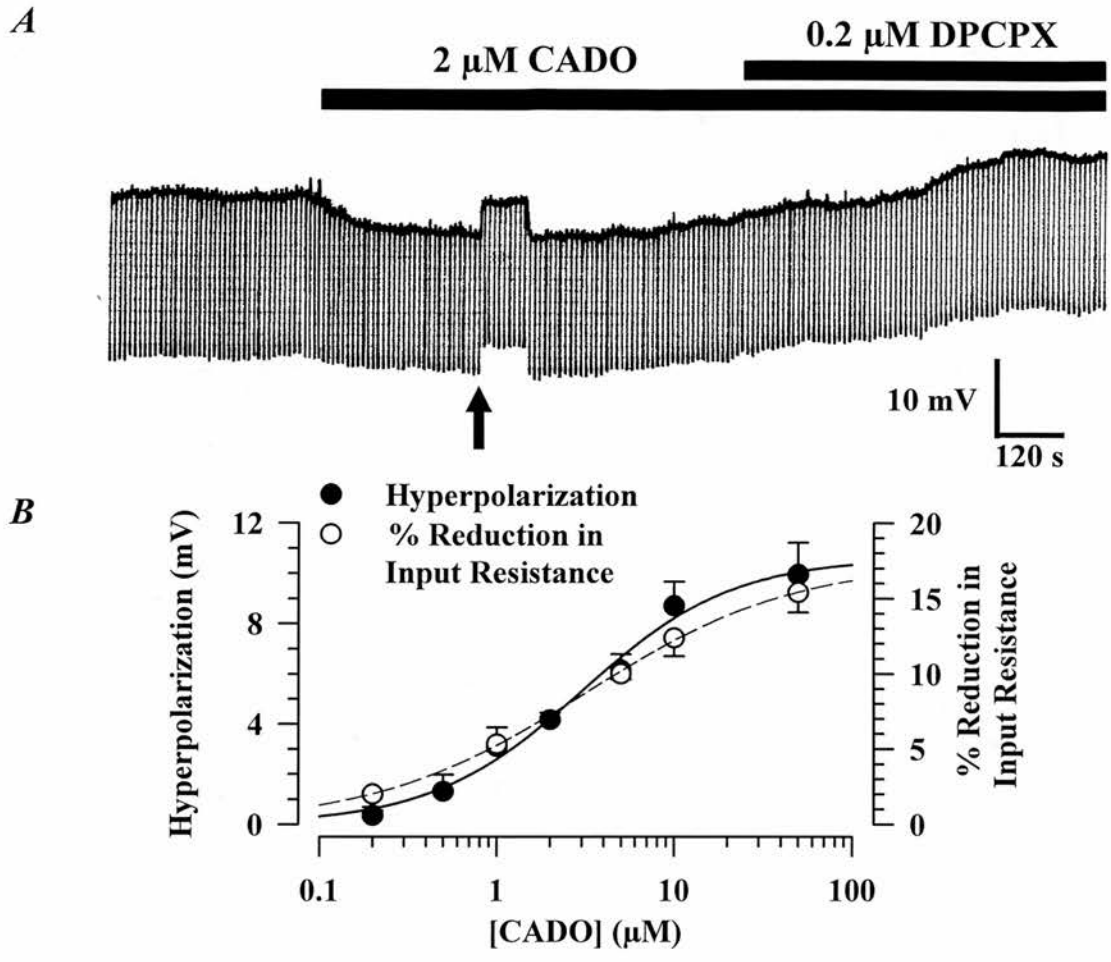

Figure 5.1. Concentration-response relationships for CADO-induced postsynaptic hyperpolarization and reduction in input resistance.

A, shows a continuous chart record to illustrate the effects of CADO and DPCPX on passive membrane properties of a CA1 neurone. The trace shows the membrane potential (thick line) and hyperpolarizing voltage responses (downward deflections) of the cell to constant current steps (-0.3 nA, 300 ms). Note (i) that CADO caused a hyperpolarization and reduction in input resistance that was completely reversed by DPCPX and (ii) that in the presence of DPCPX the membrane potential became more depolarized than that before any drug treatment, presumably indicating the presence of an endogenous adenosine tonus in the slice. The initial membrane potential of this neurone was -62 mV. *B*, shows plots of the magnitude of CADO-induced postsynaptic hyperpolarization, from a starting membrane potential of between -62 and -64 mV, *versus* concentration of CADO (●; data from 14 neurones). Superimposed on this is a plot of the reduction in input resistance of the cell by CADO *versus* concentration of CADO (○; data from 14 neurones). In this and subsequent figures all data (Y) were fitted to the logistic expression $Y = M(X^P/[X^P + K^P])$ where X is the concentration of CADO, M the maximum effect, K is the IC_{50} or EC_{50} value and the power P determines the slope of the curve.

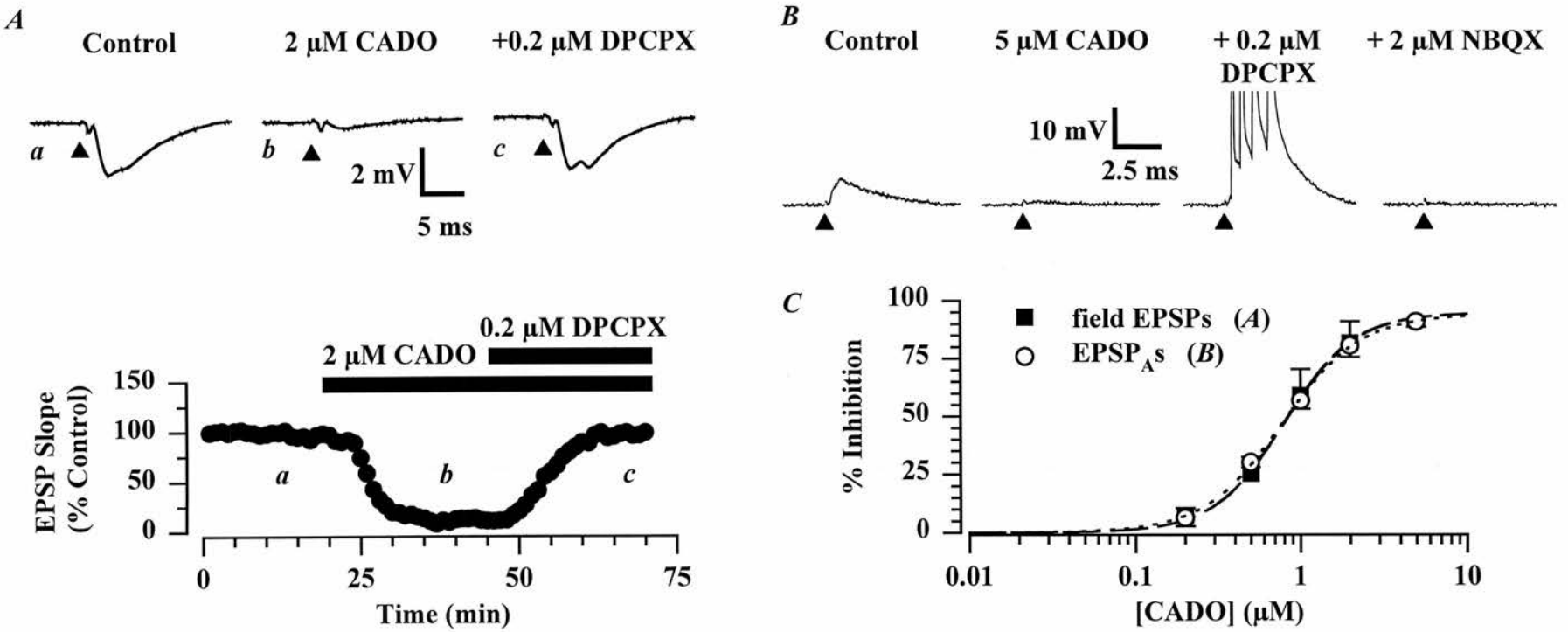

Figure 5.2. Concentration-response relationships for CADO-induced depression of glutamate receptor-mediated EPSPs.

A, shows extracellular field recorded glutamate-mediated EPSPs in control medium (*a*) in the presence 2 μM CADO (b) and in the additional presence of 0.2 μM DPCPX (*c*). The graph below shows a plot of the normalized peak amplitudes of successive EPSPs, *versus* time for a single experiment. In *B*, responses are isolated AMPA receptor-mediated EPSPs (EPSP$_A$s) recorded in the combined presence of 50 μM CGP 40116, 50 μM picrotoxin and 1 μM CGP 55845A. The traces, from left to right, are representative EPSP$_A$s recorded in control medium, in the presence of 5 μM CADO, in the combined presence of 5 μM CADO and 0.2 μM DPCPX and following subsequent application of 2 μM NBQX. Note that DPCPX reversed the depression of EPSP$_A$s and field EPSPs caused by CADO. In addition, field EPSPs and EPSP$_A$s following addition of DPCPX were larger than control responses. Finally, EPSP$_A$s were abolished by NBQX. In *A* and *B*, each trace is an average of four successive responses evoked 15 s apart. The membrane potential of the neurone was -70 mV. *C*, shows plots of the CADO-induced percentage inhibition of extracellular glutamate field EPSPs (■; data from four slices), and AMPA receptor-mediated EPSPs (○; data from four neurones), *versus* concentration of CADO. Each point is the mean value obtained from 3-4 hippocampal slices (error bars represent the S.E.M.). Data were fitted to the logistic expression described in Fig. 5.1.

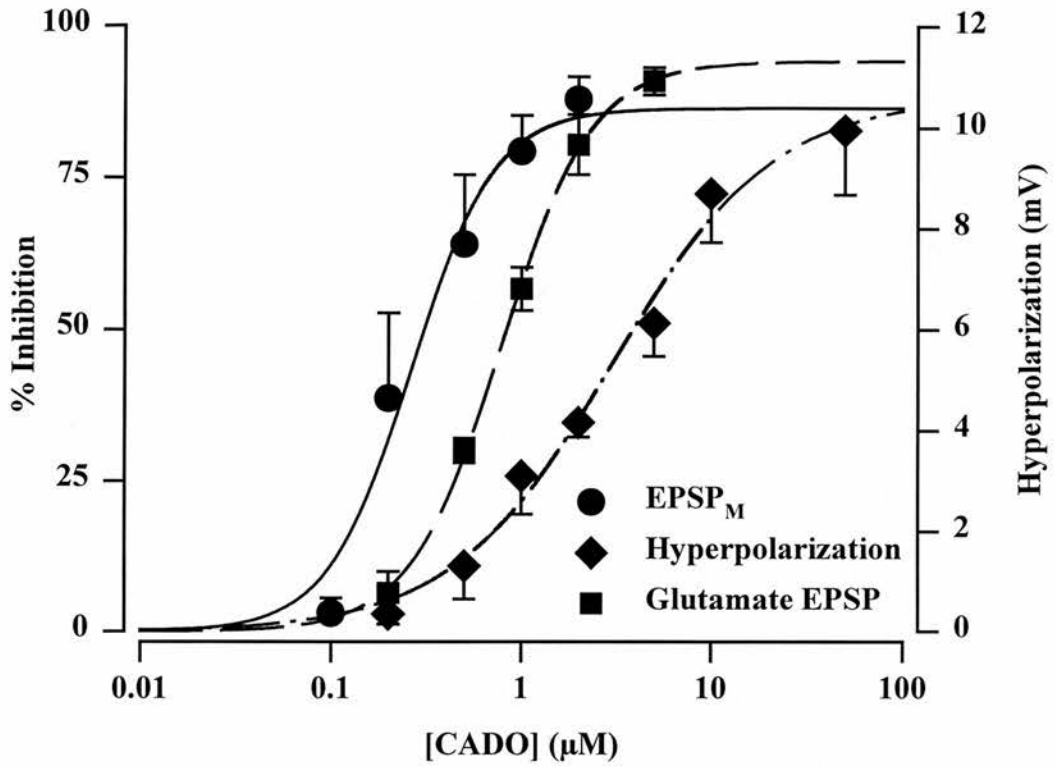

Figure 5.3. Comparison of the concentration-response relationships for CADO-induced depression of the $EPSP_M$ with those for postsynaptic hyperpolarization and depression of glutamate-mediated EPSPs.

This figure shows plots of the percentage inhibition of $EPSP_M$s (●; data from 19 neurones) and of AMPA receptor-mediated EPSPs (■; data from 4 cells) induced by CADO *versus* concentration of CADO. Each point for each plot is the mean value obtained from 3–9 separate neurones and the error bars represent S.E.M.s. Superimposed on these plots is a plot of the magnitude of CADO-induced postsynaptic hyperpolarization, from a starting membrane potential of between -62 and -64 mV, *versus* concentration of CADO (◆; data from 14 neurones). All data were fitted to the logistic expression described in Figure 5.1.

5.2.1.3. Comparison of the effects of adenosine receptor activation

As illustrated in Figure 5.3, CADO was more potent at inhibiting the $EPSP_M$ than it was at inhibiting $EPSP_A$s or causing postsynaptic hyperpolarization. The respective EC_{50} values for the depression of $EPSP_M$s, $EPSP_A$s and postsynaptic hyperpolarization were 0.3 μM, 0.8 μM and 3.0 μM (Fig. 5.3). In addition, the logistic fit for the depressant action of CADO on the $EPSP_M$ more closely paralleled that for its presynaptic depressant action on glutamate-mediated EPSPs.

5.2.2. The Effects of Adenosine Receptor Activation on Postsynaptic Responses to Carbachol

If CADO was acting presynaptically to depress both the $EPSP_M$s and reduction in SFA evoked by sub-threshold stimulation, then it should not affect the equivalent postsynaptic mAChR-mediated responses evoked by CCh. To maximize the probability of observing an effect of CADO on CCh-induced postsynaptic responses concentrations of CCh were used that (i) were close to the EC_{50} values reported for causing postsynaptic depolarization and reductions in SFA (Madison *et al.* 1987) and (ii) produced responses similar to those evoked by afferent stimulation (see Figs. 3.6 & 3.11). In addition, concentrations of CADO were used that were near maximal for inhibiting the $EPSP_M$ and the reduction in SFA evoked by sub-threshold stimulation (see Figs. 5.3 & 4.8).

5.2.2.1. The effect of adenosine receptor activation on carbachol-induced depolarization and increase in input resistance

In a first series of experiments, the effect of CADO on the postsynaptic depolarization and increase in input resistance evoked by brief bath applications of CCh (3 μM for 30–60 s) were investigated. In four neurones, repeated applications of CCh caused consistent and reversible depolarizations that were associated with increases in input resistance (Fig. 5.4). In these same neurones addition of CADO (1 μM) caused a hyperpolarization (1–5 mV) that was associated with a small decrease in input resistance (4–7 %) (Fig. 5.4*A*).

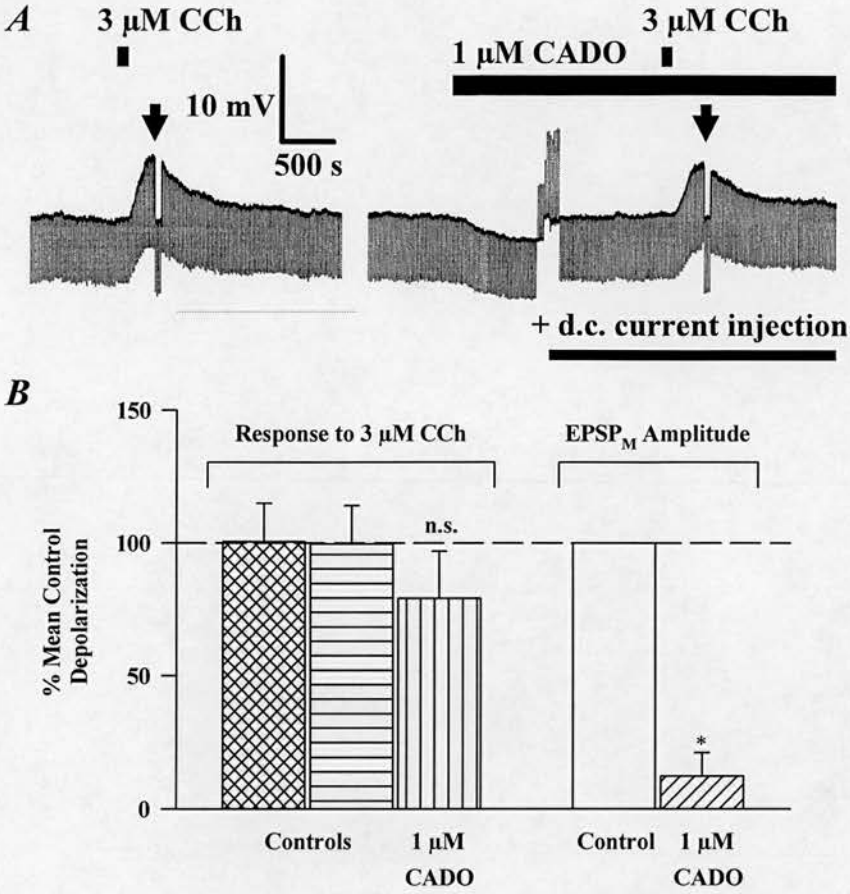

Figure 5.4. The effects of CADO on postsynaptic depolarizing responses evoked by carbachol: A comparison with the effect on EPSP$_M$s.

A, shows the effect of CADO (1 μM) on the change in membrane potential and input resistance evoked by CCh (CCh; 3 μM). The trace is a chart record of membrane potential and cell input resistance, as described in Figure 5.1, illustrating the effects of CCh on these parameters prior to and following CADO application. The bar below the chart record indicates where DC injection was used, in the presence of CADO, to restore the membrane potential of the cell to that prior to CADO application. This was kept constant for the period indicated by the length of the bar except for when the increase in input resistance evoked by CCh was measured. The upward deflections just prior to, and just after, the point where DC was first applied represent voltage responses to positive constant current steps (+0.3 nA, 300 ms). The bars above the chart record represent the times for which drugs were applied. The initial membrane potential of this neurone was -64 mV. *B*, On the left-hand side of the bar graph pooled data for the peak amplitude of the two CCh (3 μM)-induced depolarizations prior to the application of CADO, and one in the presence of 1 μM CADO, are plotted as a percentage of the mean value for the responses prior to CADO application ($n = 4$). On the right-hand side of the bar graph pooled data for the peak amplitude of the EPSP$_M$ evoked in the presence of 1 μM CADO is plotted as a percentage of control ($n = 8$; see Fig. 4.2). Note that the same concentration of CADO depressed the EPSP$_M$ to a greater extent than it did the CCh-induced depolarization. The small reduction in the size of the CCh-induced depolarization in the presence of CADO is not statistically significant and can be explained largely on the basis of the decrease in input resistance induced by CADO in this group of cells. In contrast, CADO significantly depressed the EPSP$_M$ ($P < 0.05$).

However, CADO had no significant effect on the CCh-induced depolarizations and increases in input resistance ($n = 4$; $P > 0.05$; Fig. 5.4*B*). In five other neurones these postsynaptic effects of CCh were abolished by atropine (1–5 μM; not illustrated). These results contrast with those for the $EPSP_M$ which was depressed significantly by both CADO (1 μM, $P < 0.05$; Fig. 5.4*B*) and atropine (1 μM, $P < 0.05$; Fig. 3.8).

5.2.2.2. The effect of adenosine receptor activation on carbachol-induced inhibition in spike frequency adaptation

In a second series of experiments, the effects of CADO and CCh on SFA evoked in response to a depolarizing current step were investigated. CCh (0.5 μM) caused a small depolarization and a reduction in SFA that was reversible on washout ($n = 5$; Fig. 5.5*A* & 3.11). Subsequent addition of CADO (5 μM) slightly enhanced the level of SFA *per se*, in agreement with previous studies (Haas & Greene, 1984), but did not significantly affect the reduction in SFA induced by a second application of CCh ($n = 3$; Fig. 5.5). In contrast, atropine (1 μM) abolished the CCh-induced reduction in SFA ($n = 5$; data not shown). These results differ from those in which SFA was reduced using sub-threshold stimulation in that the latter was abolished by both CADO (5 μM; Fig. 5.5) and atropine (1 μM; Fig. 3.11).

5.2.3. The Mechanism of Action of Adenosine

5.2.3.1. Involvement of 4-AP sensitive potassium channels

As the previous results suggest a presynaptic locus for these A_1R mediated effects, this raises the possibility of an effect on presynaptic K^+ channels that subsequently affects the release of ACh. To investigate this possibility, the K^+ channel blocker 4-aminopyridine (4-AP) was used. 4-AP inhibits the ability of adenosine to inhibit glutamate release in the guinea-pig hippocampus (Okada & Ozawa, 1980). 4-AP (100 μM) increased the amplitude of $EPSP_M$s from 2.1 ± 0.3 mV to 21.6 ± 1.5 mV ($n = 4$; Fig. 5.6*A*). In the presence of 4-AP (100 μM), CADO (1 μM) depressed the amplitude of $EPSP_M$s by 54 ± 12 % ($n = 4$; Fig. 5.6). This contrasts with a depression of 84 ± 5 % for depression of $EPSP_M$s by 1 μM CADO in the absence of 4-AP (see Fig. 5.4*B*).

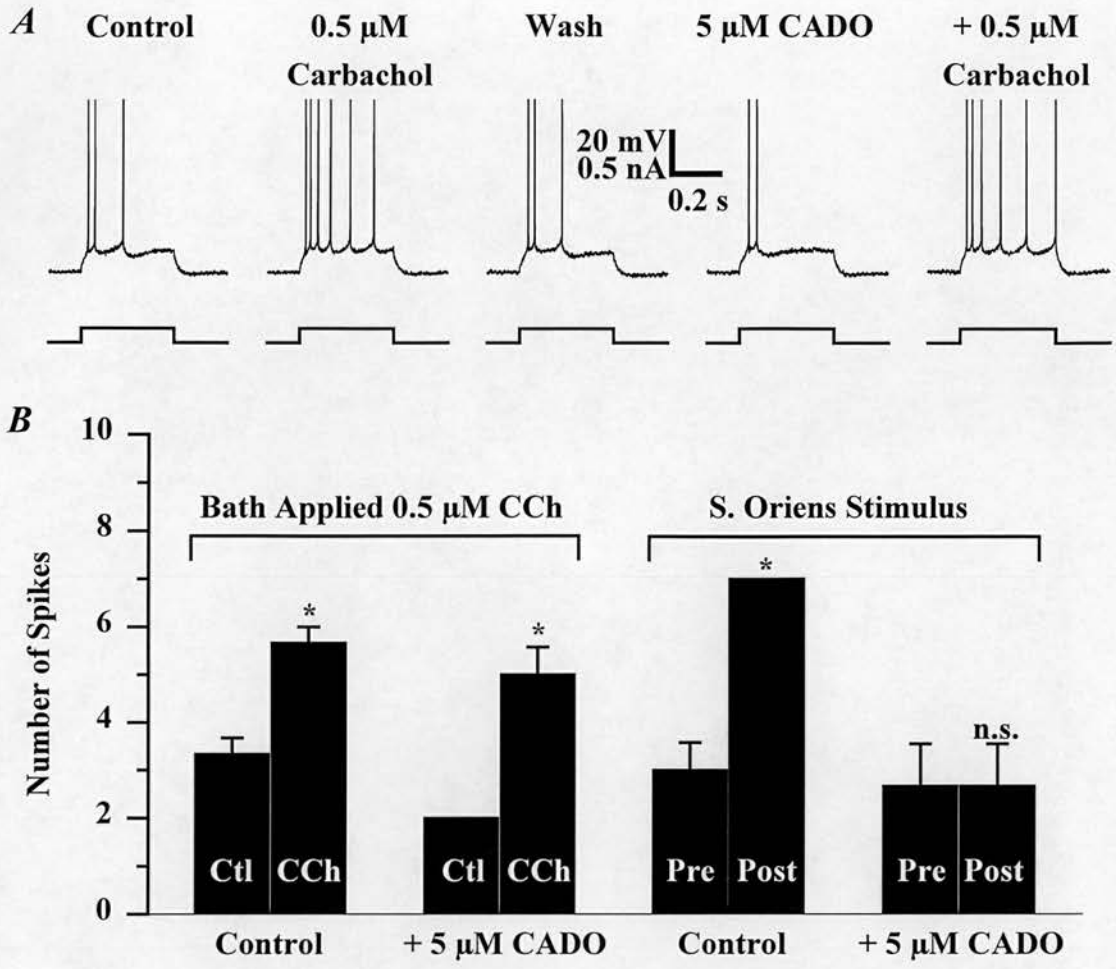

Figure 5.5. The effects of CADO on postsynaptic reduction in spike frequency adaptation by carbachol: A comparison with the effect on stimulation evoked reduction in spike frequency adaptation.

A, shows, from left to right, responses evoked by a depolarizing current step (+0.2 nA, 400 ms) in control medium, in the presence of CCh (0.5 µM), following washout, in the presence of CADO (5 µM) and in the combined presence of CADO (5 µM) and CCh (0.5 µM). The membrane potential of the cell was maintained at -66 mV throughout this experiment by injecting DC through the recording electrode to compensate for the hyperpolarizing and depolarizing effects of CADO and CCh, respectively. The trace at the bottom of the figure represents the time during which positive current steps were injected via the recording electrode. Note that CCh reduced SFA evoked during the depolarizing step and that this was unaffected by CADO. *B*, shows a bar graph in which the left-hand side represents pooled data for the number of action potentials fired during a depolarizing step in the absence (Ctl) and presence of 0.5 µM carbachol (CCh) plotted for control medium and for medium containing 5 µM CADO ($n = 3$). The right-hand side shows pooled data for the number of action potentials fired during a depolarizing step 1.0 s before (Pre) and 2.0 s after (Post) a sub-threshold stimulus in the absence and presence of 5 µM CADO ($n = 3$) (See Fig. 4.7). Statistical significance was tested for CCh *versus* control (Ctl) and Post *versus* Pre data for each of the paired data sets. Statistical significance was assigned where $P < 0.05$. Note that CADO completely inhibited the increase in number of action potentials fired following sub-threshold stimulation without affecting the increase evoked by CCh application.

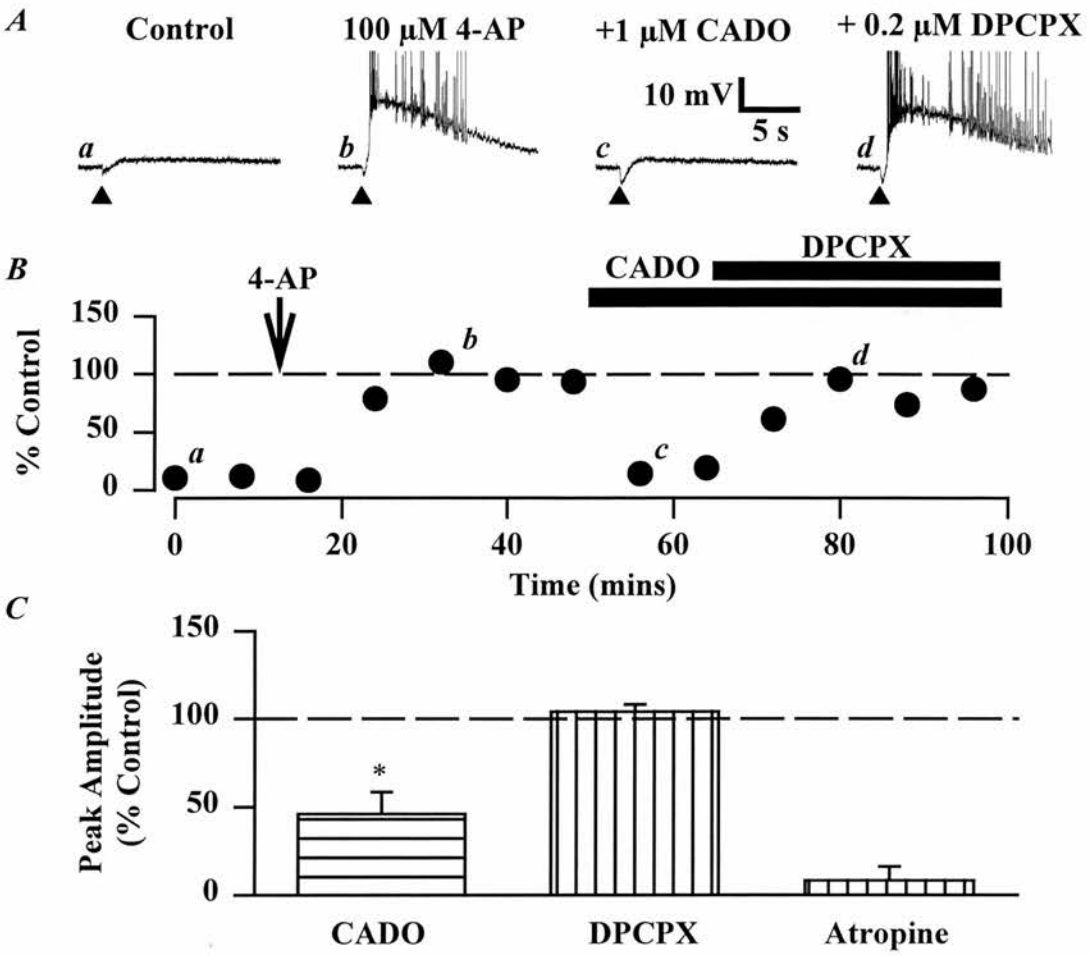

Figure 5.6. The effects of CADO on $EPSP_M$s in the presence of 4-AP.

In *A*, synaptic traces are isolated $EPSP_M$s recorded in control medium (containing ionotropic glutamate and GABA receptor antagonists) (*a*), in the presence of 100 µM 4-AP (*b*), in the presence of 100 µM 4-AP + 1 µM CADO (*c*) and in the additional presence of 0.2 µM DPCPX (*d*). The graph (*B*) shows a plot of the peak amplitudes of successive $EPSP_M$s normalized to the mean peak amplitude of the three $EPSP_M$s prior to CADO application in the presence of 100 µM 4-AP *versus* time, for a single experiment. 1 µM CADO and 0.2 µM DPCPX were applied for the times indicated by the bars. The points marked *a*, *b*, *c* and *d* refer to the synaptic traces illustrated above the graph. *C*, is a bar graph illustrating pooled data for the amplitudes of $EPSP_M$s in the presence of 1 µM CADO (n = 4), 1 µM CADO + 0.2 µM DPCPX (n = 2) and in the additional presence of 1 µM atropine (n = 2) all in the presence of 100 µM 4-AP. Amplitudes are expressed as a percentage of the amplitude of $EPSP_M$s in the presence of 100 µM 4-AP. The values plotted were calculated as described in Figure 4.4*E*. Note that CADO reversibly inhibited $EPSP_M$s in the presence of 4-AP. The membrane potential of the neurone was -64 mV.

The depression of $EPSP_M$s in the presence of 4-AP was subsequently reversed by DPCPX (0.2 μM; $n = 2$; Fig 5.6) and the responses in the presence of both CADO and DPCPX were inhibited by atropine (1 μM; $n = 2$; Fig 5.6).

5.2.3.2. Involvement of the cAMP/adenylate cyclase system

Classically, A_1Rs have been shown to be negatively coupled to AC. To assess the involvement of a reduction in intracellular cAMP in the inhibitory response to CADO, the effects of the membrane permeable analogue of cAMP, 8-bromo cAMP (8-Br cAMP) were also investigated. The rationale behind these experiments was that the presence of 8-Br cAMP should compensate for any reduction in cAMP. 8-Br cAMP (1 mM) inhibited SFA but had no significant effect on $EPSP_M$s ($n = 5$) in agreement with previous studies (Madison *et al.* 1987). In addition, 8-Br cAMP (1 mM) reversed or inhibited the depression of $EPSP_M$s by CADO (1 μM; $n = 4$; Fig. 5.7*A* & *B*).

5.3. DISCUSSION

5.3.1. Locus of the Adenosine Receptor-Mediated Depression of the $EPSP_M$

As A_1Rs are found in abundance both pre- and post-synaptically in the CA1 region of the hippocampus, it is possible that adenosine inhibits mAChR-mediated postsynaptic responses by activation of one or both populations of these receptors. Presynaptic inhibition of transmitter release is thought to be the primary mechanism responsible for A_1R inhibition of synaptic transmission at hippocampal glutamatergic synapses. Three main lines of evidence have led to this hypothesis,

i) adenosine increases paired pulse facilitation (Dunwiddie & Haas, 1985),

ii) adenosine inhibits evoked transmitter release (Cunha *et al.* 1994), and

iii) adenosine reduces quantal content without reducing quantal size (Lupica & Dunwiddie, 1992).

In the present study, however, it was not possible to use a paired pulse protocol due to the fatiguing of the response on stimulating more often than every 8 min

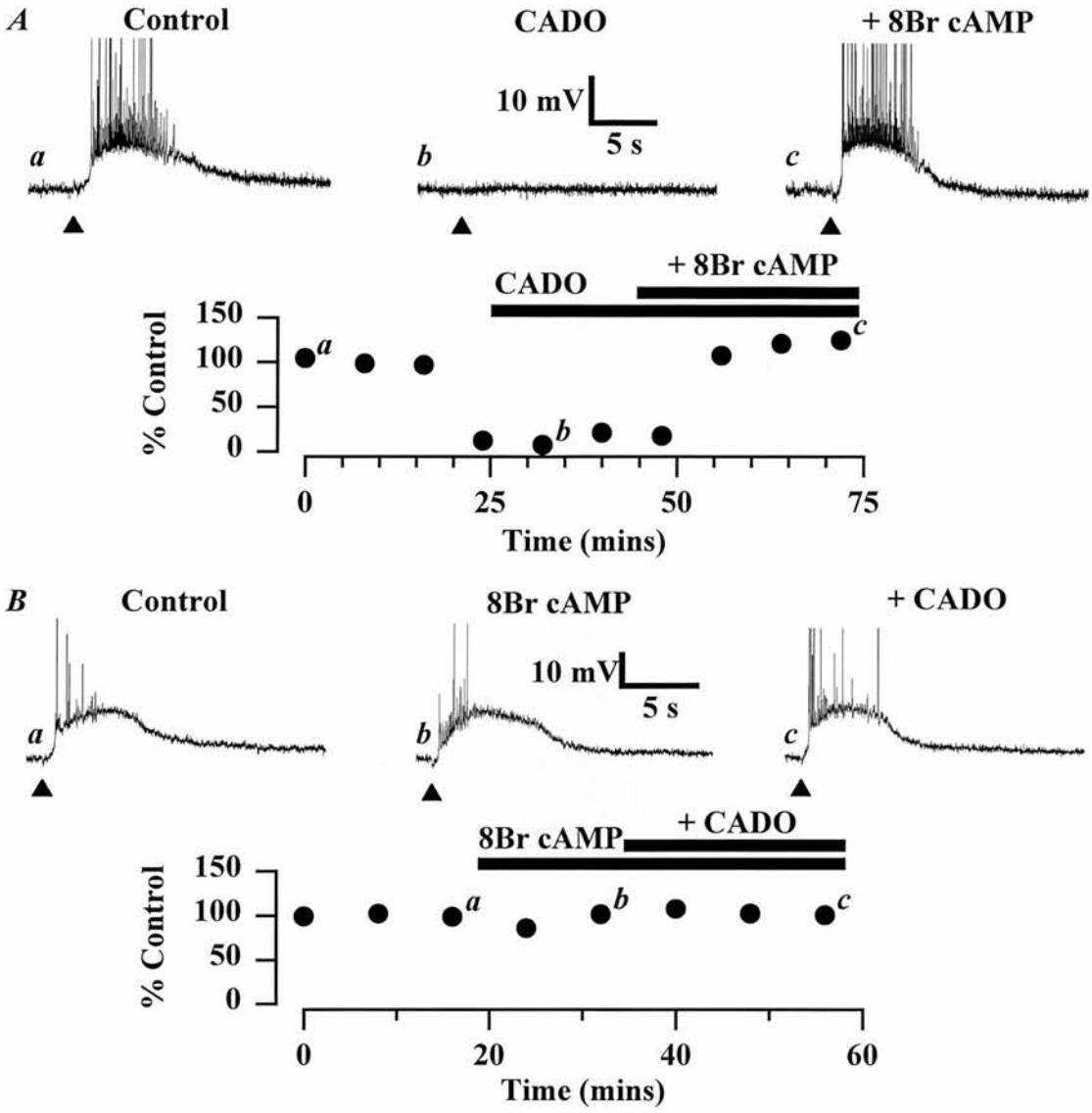

Figure 5.7. The effects of CADO on $EPSP_M$s in the presence of 8-Br cAMP.

In *A*, synaptic traces are isolated $EPSP_M$s recorded in control medium (*a*), in the presence of 1 μM CADO (*b*) and in the additional presence of 1 mM 8-Br cAMP (*c*). The graph below shows a plot of the peak amplitudes of successive $EPSP_M$s, normalized to the mean peak amplitude of the three $EPSP_M$s prior to CADO application, *versus* time for a single experiment. *B*, shows a similar experiment to that shown in *A* in which synaptic traces are isolated $EPSP_M$s recorded in control medium (*a*), in the presence of 1 mM 8-Br cAMP (*b*) and in the additional presence of 1 μM CADO (*c*). The graph below is constructed in the same way as that shown in *A*. In both graphs in *A* and *B*, 1 mM 8-Br cAMP and 1 μM CADO were applied for the times indicated by the bars and the points marked *a*, *b* and *c* refer to the synaptic traces illustrated above each graph. Note that CADO inhibited $EPSP_M$s and that 8-Br cAMP reversed or prevented this inhibition. The membrane potential of both neurones was -63 mV.

(see section 3.3.2). Likewise, it was not possible to investigate quantal content, as mAChR-mediated synaptic transmission is non-quantal. That the concentration-response relationship for the CADO-induced depression of the $EPSP_M$ most closely paralleled that for the depression of the $EPSP_A$, but not that for the postsynaptic hyperpolarization, indirectly favors a presynaptic locus of the A_1R-mediated depression. In this respect, at least three additional lines of evidence suggest the involvement of a presynaptic depressant mechanism:

i) CADO can depress the $EPSP_M$ even at concentrations that do not affect postsynaptic passive membrane properties (see Fig. 4.3),

ii) CADO does not significantly inhibit CCh-induced postsynaptic responses, and

iii) both the $EPSP_M$ and mAChR-mediated postsynaptic reduction of SFA evoked by sub-threshold stimulation, which result from the inhibition of distinct K^+ conductances ($I_{K(LEAK)}$ and I_{AHP}; Madison *et al.* 1987), are inhibited by CADO.

In addition, neurochemical data from both hippocampal synaptosomes and slices strongly indicate the existence of a presynaptic A_1R-mediated inhibition of evoked [^{3}H]-ACh release (Cunha *et al.* 1994). However, it is difficult to compare K^+ or electrically evoked release with synaptic release of neurotransmitter as the mechanism of synaptic release may differ. Nevertheless, *in vivo*, adenosine receptor activation has also been shown to inhibit the turnover rate of ACh (Haubrich *et al.* 1981; Murray *et al.* 1982). When taken together these data provide convincing evidence that a significant proportion of the A_1R-mediated inhibition of the $EPSP_M$ and associated reduction in SFA occurs by presynaptic inhibition of ACh release. Such a mechanism has previously been proposed for cholinergic synapses at the neuromuscular junction where activation of A_1Rs inhibits the average number of quanta of ACh released from the nerve terminal without affecting the size of each individual quantum (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975; Silinsky, 1984).

It is conceivable that adenosine may also act postsynaptically to depress mAChR-mediated postsynaptic responses. In this respect, a direct interaction between the

transduction mechanisms activated by A_1Rs and mAChRs seems unlikely, as A_1Rs classically couple to the G-protein G_i, which inhibits AC activity. In contrast, neither the $EPSP_M$ nor the mAChR-mediated reduction in SFA is affected by cAMP mimetics such as 8-Br cAMP (Madison *et al.* 1987) or antagonists of protein kinase A (Pedarzani & Storm, 1993). However, negative interactions between A_1Rs and the M_1 mAChR-mediated inhibition of I_M have been reported in the superior cervical ganglion (Connolly & Stone, 1995). Despite this, I_M is unlikely to account for the actions of synaptically activated mAChRs in the hippocampus (Madison *et al.* 1987) even though pyramidal neurones are thought to express both the M_1 and/or M_3 mAChR subtypes (Dutar & Nicoll, 1988a; Pitler & Alger, 1990; Segal & Fisher, 1992). These receptors classically couple through the pertussis-toxin insensitive $G_{q/11}$ family of G-proteins to PLC, the activity of which can, in some cells, e.g. aorta, be inhibited by A_1Rs. However, it is unclear whether activation of PLC fully accounts for mAChR-mediated depolarization and reductions in SFA in the hippocampus (Muller & Misgeld, 1986; Dutar & Nicoll, 1988a; Colino & Halliwell, 1993).

Adenosine (P_1) receptor activation has been shown to modulate the effects of CCh on field recorded synaptic potentials in area CA1 of the rat hippocampus (Brooks & Stone, 1980). However, these effects are difficult to interpret because it is not possible to account for the intracellular hyperpolarizing effects of adenosine. Whatever the case, the inability of CADO to abolish both CCh-induced depolarization and reduction in SFA would suggest that this mechanism or, for example, a membrane delimited interaction between the A_1R-mediated signal transduction mechanism(s) and mAChR coupled K^+ conductances, are unlikely to account for the depressant effects of A_1Rs on the $EPSP_M$ and stimulation-evoked reduction in SFA (see Fig. 5.5). Nevertheless, adenosine, by activating postsynaptic K^+ conductances, will restrict the magnitude of the $EPSP_M$ recorded at the soma, to some extent, by the shunting of membrane currents as well as hyperpolarization towards the reversal potential of the $EPSP_M$ (Cole & Nicoll, 1984a).

5.3.2. Mechanism of Action of Adenosine Receptor Activation

Having established a likely locus for the A_1R-mediated effects on mAChR-mediated

responses as presynaptic, the mechanism for this effect must involve either

i) an indirect inhibition of presynaptic excitability,

ii) an effect on local network function, or

iii) the modulation of transmitter release.

Firstly, an indirect action on the excitability of the presynaptic neurone can be excluded because of the preparation used. In the hippocampal slice the septohippocampal axons projecting from medial septum have been severed. As such, is not possible for A_1Rs to affect the driving force of the presynaptic septal cholinergic neurone. With respect to the effects on local network function, although mAChR-mediated synaptic responses are likely to be monosynaptic and septal afferents also innervate interneurones in the hippocampus, evoked responses in these experiments were isolated from ionotropic GABA and glutamate receptor-mediated effects using antagonists at these receptors. These factors suggest that effects of A_1R activation on local network function is unlikely.

In terms of a modulation of transmitter release, activation of presynaptic K^+ channels has been proposed as a mechanism for the inhibition of transmitter release by adenosine at glutamatergic synapses (Dunwiddie, 1990). In neurochemical release studies, however, there are conflicting reports regarding the involvement of inhibition of K^+ channels in the inhibitory effect of A_1R activation on hippocampal ACh release (Benishin, 1990; Fredholm, 1990; 1993). Inhibition of 4-AP sensitive K^+ channels facilitated $EPSP_M$s but did not completely block the inhibition of $EPSP_M$s by CADO. This may suggest that the concentration of 4-AP was not sufficient to inhibit the particular K^+ channel involved, however, a high concentration of 4-AP was used (100 μM). This concentration of 4-AP has previously been shown to be sufficient to inhibit the presynaptic inhibitory action of mGluRs (Sladeczek *et al.* 1993). Alternatively, this may suggest that 4-AP sensitive K^+ channels are not, exclusively at least, involved in the inhibitory effects of adenosine on $EPSP_M$s. These data are in contradiction to adenosine receptor-mediated inhibition of glutamatergic synaptic transmission in the hippocampus and olfactory cortex, which is abolished by 4-AP (Okada & Ozawa, 1980; Scholfield & Steel, 1988). A potential

problem with the use of K^+ channel blockers in such experiments is that they increase the magnitude of synaptic responses alone through inhibition of presynaptic K^+ channels. Although this did not abolish the ability of A_1R activation to inhibit $EPSP_M$s, it was noticeable that the magnitude of depression produced by adenosine was less in the presence of 4-AP than in control experiments. It is possible that this reduction in the efficacy of CADO was due to increased intracellular Ca^{++} in the presynaptic terminal swamping the release machinery, thus rendering the release process unresponsive to inhibition. Other studies, however, have excluded this mechanism, for example, at glutamatergic synapses (Scholfield & Steel, 1988). As such, a contribution of 4-AP-sensitive K^+ channels to the reduction in the efficacy of CADO must be considered. This can be further investigated by

i) in the presence of 4-AP increasing the concentration of Mg^{++} to reduce the release of transmitter to control levels, or

ii) reducing the stimulus intensity in the presence of 4-AP, to reduce the amplitude of the $EPSP_M$.

In addition to the activation of presynaptic K^+ conductances, inhibition of Ca^{++} currents or a direct inhibition of the Ca^{++}-sensitive release machinery (Silinsky, 1984; 1986) may modulate transmitter release. These possible mechanisms have gained some support over the past 12 years and have been reviewed in detail for glutamate synapses (Silinsky, 1986; Fredholm & Dunwiddie, 1988; Thompson *et al.* 1993; Wu & Saggau, 1997). It is unclear which Ca^{++} channels mediate ACh release. Future studies may address this issue and ascertain whether ACh release, like glutamate release, is mediated by N- and Q- and/or P-type Ca^{++} channels (Luebke *et al.* 1993; Wheeler *et al.* 1994) and whether inhibition of ACh release by A_1R activation is also mediated by effects on N- and/or Q-type Ca^{++} channels, as at glutamate synapses (Wu & Saggau, 1994).

Finally, inhibition of release machinery is a possibility. In neurochemical release studies it has been suggested that the A_1R-mediated inhibition of ACh release is unaffected by ω-conotoxin or intracellular Ca^{++} chelation, suggesting that inhibition of release is upstream of Ca^{++} entry and may in fact be a direct effect on the release

mechanism itself (Fredholm, 1993). It is unclear, however, whether ACh release in such release studies is modulated by the same release processes as synaptic ACh release.

Whatever the case it is clear that 8-Br cAMP occludes the effect of CADO to depress the $EPSP_M$. There is only limited evidence for the involvement of AC in the reduction of transmitter release by adenosine (McCabe & Scholfield, 1985). Data presented in this chapter suggests, controversially, that cAMP is involved in this A_1R-mediated inhibition of mAChR-mediated responses. That 8-Br cAMP reversed or prevented the inhibition of $EPSP_M$s by CADO suggests that it is counteracting an A_1R-mediated reduction in transmitter release by increasing intracellular cAMP turnover. This is in direct contradiction to results obtained in neurochemical release studies (Dunér-Engström & Fredholm, 1988). However, this result may be misleading, as 8-Br cAMP will activate PKA which could phosphorylate Ca^{++} and K^+ channels presynaptically, thus altering their function. This is unlikely, however, as 8-Br cAMP did not have any effect on $EPSP_M$s suggesting that the release process was unaffected. Nevertheless, the possibility remains that the resulting activation of PKA alters the function of other transduction processes exclusively involved in the A_1R-mediated modulation of ACh release. In addition, 8-Br cAMP has been reported to have a direct agonist effect on A_1Rs (Dolphin *et al.* 1986). If 8-Br cAMP were acting as an agonist at A_1Rs, it would have been expected to affect $EPSP_M$s directly. However, if 8-Br cAMP did not affect $EPSP_M$s it may alternatively act as a partial agonist at A_1Rs.

The apparent contradictions in this field of research have led to complicated theories regarding the mechanism(s) of adenosine receptor-mediated inhibition of transmitter release. It has been speculated that there can be no unifying mechanism to explain A_1R-mediated inhibition of transmitter release (Starke, 1987).

5.4. SUMMARY

Experiments were conducted to assess the pre- or post-synaptic locus of the A_1R-mediated inhibition of $EPSP_M$s. The concentration-response relationship for the inhibition of $EPSP_M$s more closely resembled that for the presynaptic depression of

glutamate receptor-mediated EPSPs, indirectly suggesting a presynaptic locus for the inhibition of $EPSP_M$s by CADO. Postsynaptic depolarization and inhibition of SFA evoked by CCh were not significantly affected by CADO at concentrations that significantly depressed cholinergic synaptic responses, suggesting a presynaptic locus for the effect of CADO. In addition, the mechanism of action of CADO appears to be partially dependent on 4-AP-sensitive K^+ channels and may involve an inhibition of cAMP turnover. As such, the A_1Rs that mediate the inhibition of cholinergic synaptic transmission are located predominantly presynaptically and may act via a cAMP-dependent mechanism.

CHAPTER 6

$GABA_B$ RECEPTOR MEDIATED MODULATION OF *MUSCARINIC* RECEPTOR MEDIATED RESPONSES

6.1. INTRODUCTION

Chapters 4 and 5 of this thesis described the modulation of synaptically evoked cholinergic responses by adenosine receptor activation. In these studies, cholinergic responses were isolated from ionotropic glutamate and GABA receptor-mediated effects using antagonists at these receptors. Inhibition of these receptor systems could, in itself, affect the transmission of cholinergic synaptic responses. The purpose of this chapter therefore was to assess this possibility. Of the receptors inhibited by this cocktail of antagonists, the $GABA_B$ receptor has most in common with A_1Rs. Thus, $GABA_B$ receptor activation leads to activation of similar transduction mechanisms to A_1Rs i.e. activation of G_i/G_o subtypes of G-protein (Dutar & Nicoll, 1988b; Trussel & Jackson, 1987). In addition, both A_1Rs and $GABA_B$ receptors modulate glutamate synaptic transmission in the hippocampus in similar ways (e.g. Thompson *et al.* 1992; Olpe *et al.* 1982). Evidence of a $GABA_B$ receptor-mediated inhibition of cholinergic synaptic transmission, however, is limited. In this respect, the $GABA_B$ receptor agonist (-)-baclofen has been shown to inhibit ACh release in the SCG (Brown & Higgins, 1979) and in the superior colliculus (Wichmann *et al.* 1987). That said, experiments were conducted to ascertain whether $GABA_B$ receptor activation may also inhibit cholinergic synaptic transmission in hippocampal area CA1. As such, experiments were conducted to investigate the effects of GABA receptor activation on cholinergic synaptic transmission and to compare these effects with those of adenosine.

6.2. RESULTS

6.2.1. THE EFFECTS OF GABA RECEPTOR ACTIVATION ON THE $EPSP_M$.

Initially the effect of GABA receptor activation on $EPSP_M$s, evoked in CA1 pyramidal neurones, was investigated. The selective $GABA_B$ receptor agonist (-)-

baclofen (5–10 μM) caused a depression of the $EPSP_M$ that was maintained for the period of the agonist application ($n = 5$; Fig. 6.1). In addition, in 2 out of 3 cells, 5 μM (-)-baclofen occluded the $IPSP_B$ that preceded the $EPSP_M$. At 5 μM, the depressant action of (-)-baclofen on the $EPSP_M$ was invariably accompanied by postsynaptic hyperpolarization (5–7 mV) which was routinely compensated for using positive current injection. The maximal inhibition of $EPSP_M$s by 5 μM (-)-baclofen was 88 %.

To confirm the pharmacological classification of the GABA receptor mediating the response to (-)-baclofen, the effect of the selective $GABA_B$ receptor antagonist CGP 55845A was also tested. CGP 55845A (1 μM) antagonized the inhibition caused by 5–10 μM (-)-baclofen in all cells tested (Fig. 6.1) such that, in the presence of both (-)-baclofen and CGP 55845A responses were 121 ± 19 % of mean control amplitudes prior to the application of (-)-baclofen ($n = 5$). In addition, CGP 55845A (1 μM) reversed the hyperpolarization caused by (-)-baclofen (5 μM).

The action of CGP 55845A alone was tested to investigate the possibility that endogenously released GABA might inhibit $EPSP_M$s under control experimental conditions. CGP 55845A (1 μM) alone caused a large but variable increase in the size of $EPSP_M$s, which had a mean control amplitude of 3.0 mV, while completely inhibiting the $IPSP_B$. As such, the peak amplitude of the $EPSP_M$ in the presence of CGP 55845A was 253 ± 74 % that of control ($n = 3$; Fig. 6.2).

6.2.2. Effects of a GABA Uptake Inhibitor on the $EPSP_M$

It is well established that synaptically released GABA activates a late $IPSP_B$ in CA1 pyramidal neurones (Dutar & Nicoll, 1988c). The increase in the $EPSP_M$ that was caused by CGP 55845A alone suggested that endogenous GABA within the slice was also capable of activating $GABA_B$ receptors to inhibit the $EPSP_M$. Therefore, the next group of experiments examined whether it was possible to potentiate the effect of endogenous GABA by impairing its uptake. To do this the effects of the GABA uptake inhibitor NNC 05-0711 were investigated. NNC 05-0711 raises extracellular GABA levels by preventing the uptake of GABA (Suzdak, *et al.* 1992).

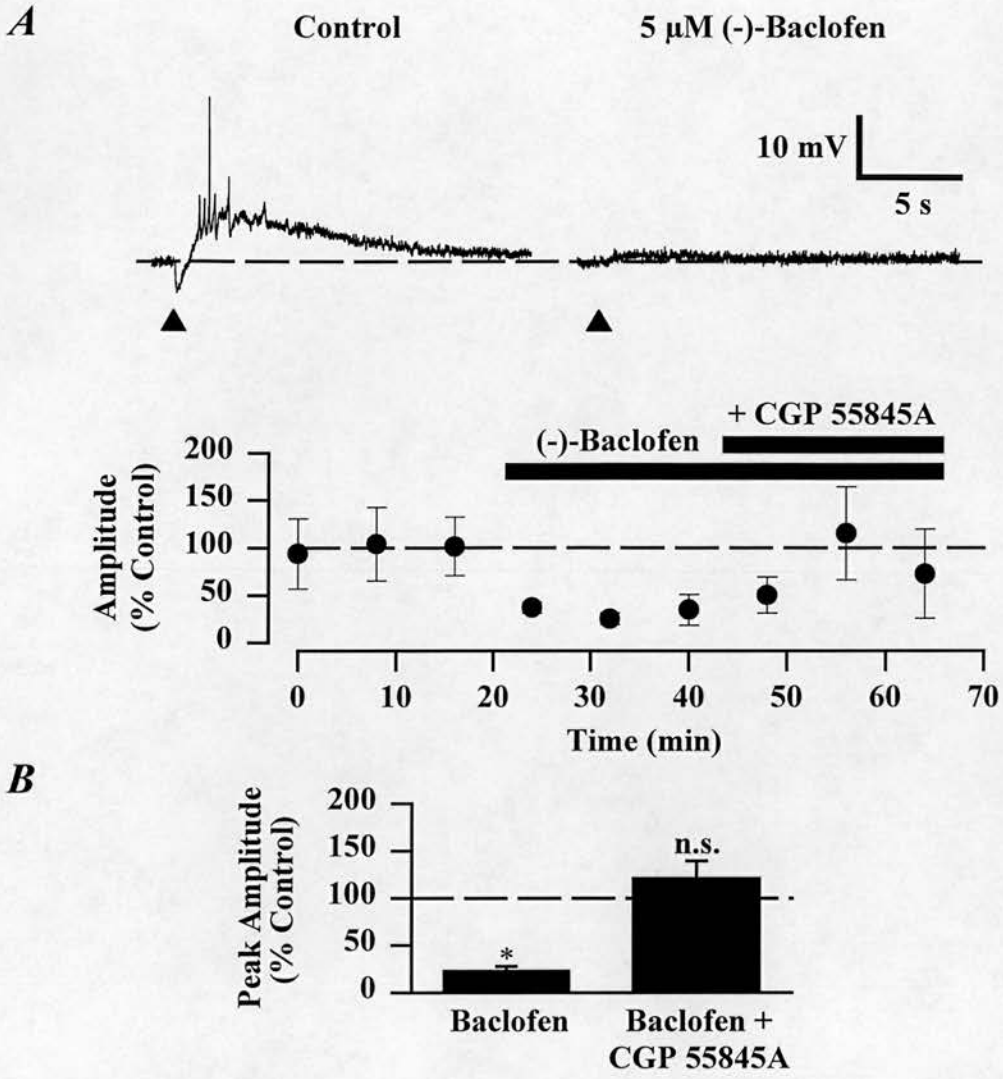

Figure 6.1. The effect of (-)-baclofen on $EPSP_M$s and the effect of subsequent addition of CGP 55845A.

In *A*, synaptic traces are $EPSP_M$s recorded in the presence of AMPA, NMDA and $GABA_A$ receptor antagonists (Control) (*a*) and in the additional presence of 5 μM (-)-baclofen (*b*). The membrane potential of the cell was -64 mV. The graph (*B*) shows a plot of the mean peak amplitudes of successive $EPSP_M$s, for pooled data, normalized to the mean peak amplitude of the five $EPSP_M$s prior to application of (-)-baclofen *versus* time to illustrate the depressant effect of (-)-baclofen on the $EPSP_M$ and its reversal by CGP 55845A. The bars above the graph indicate the duration for which the two drugs were applied. In *C*, the bar graphs illustrate pooled data for the effects of 5–10 μM (-)-baclofen ($n = 5$) and 5–10 μM (-)-baclofen + 1 μM CGP 55845A ($n = 5$) on the $EPSP_M$. The amplitudes of $EPSP_M$s are expressed as a percentage of the mean value of the control $EPSP_M$s. Note that (-)-baclofen significantly depressed the $EPSP_M$ and responses in the presence of (-)-baclofen + CGP 55845A were not significantly different from control responses. Data are means ± S.E.M.; * represents significance $P < 0.05$; n.s., not significant (compared with control). Data was obtained in conjunction with D. O. Bulters.

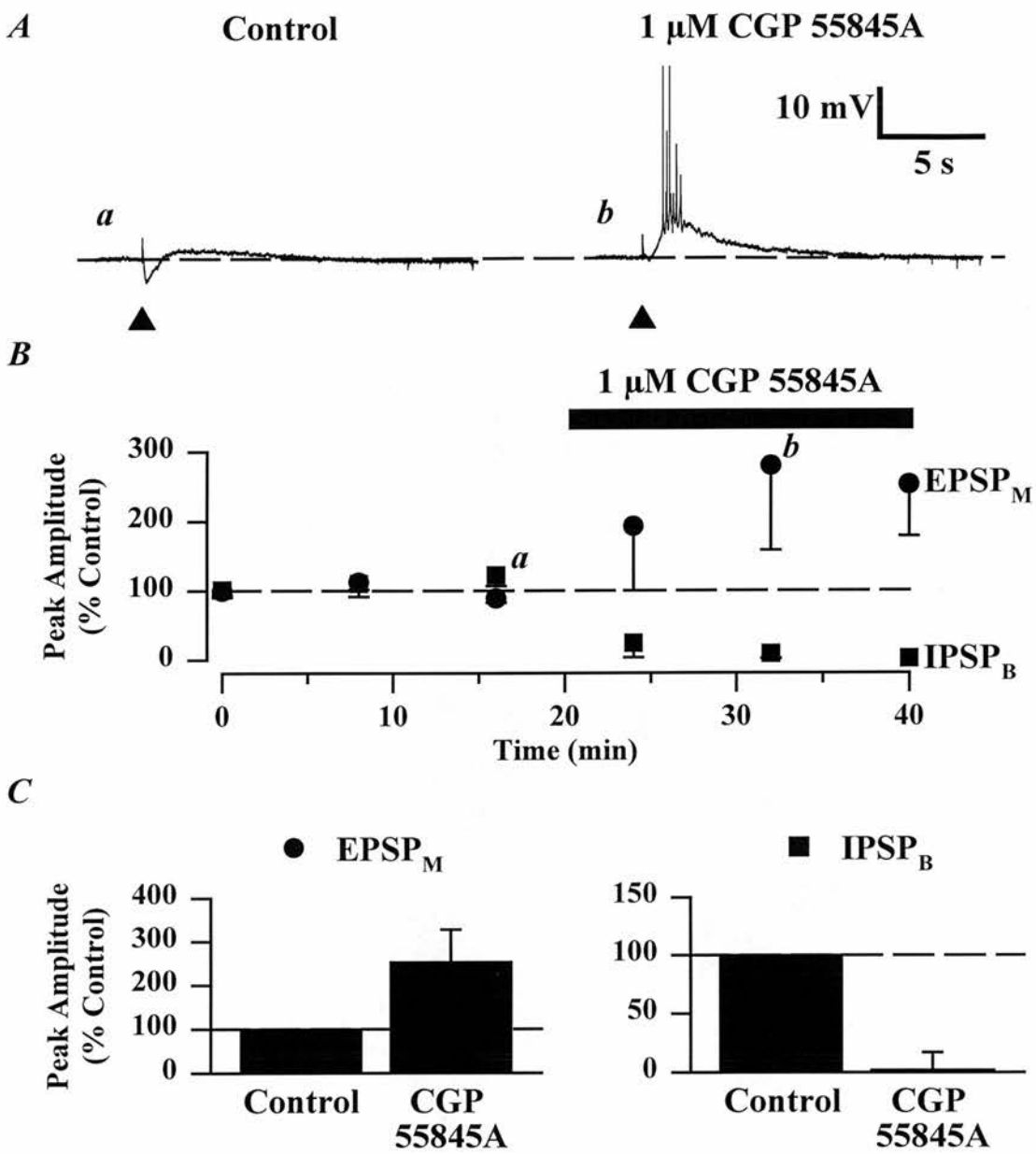

Figure 6.2. The effects of CGP 55845A alone on the $EPSP_M$ and $IPSP_B$.

In *A*, synaptic traces are representative $EPSP_M$s recorded in control (*a*) and in the presence of 1 μM CGP 55845A (*b*). Note, that following stimulation, the neurone responds with a hyperpolarization ($IPSP_B$) followed by a small $EPSP_M$. The membrane potential of this neurone was -64 mV. In *B*, the graph is similar to that illustrated in Fig. 6.1 and plots the mean peak amplitude of successive $EPSP_M$s and $IPSP_B$s, for pooled data ($n = 3$), *versus* time to illustrate the enhancement of the $EPSP_M$ and the depression of the $IPSP_B$ by CGP 55845A. The bar above the graph indicates the duration for which CGP 55845A was applied. *C*, is a bar graph illustrating pooled data for the effect of 1 μM CGP 55845A on the amplitude of the $EPSP_M$ ($n = 3$). The values plotted were calculated as described in Figure 6.1. Note that CGP 55845A abolished the $IPSP_B$ but dramatically increased the amplitude of the $EPSP_M$.

NNC 05-0711 (10 μM) increased the amplitude of $IPSP_B$s to 241 ± 38 % of control amplitudes and caused a 58 ± 10 % depression of the $EPSP_M$ (n = 4; Fig 6.3). In two of these neurones, subsequent application of CGP 55845A (1 μM) completely reversed these effects (Fig. 6.3*C*).

6.2.3. Effects of (-)-Baclofen on the Reduction in Spike Frequency Adaptation Evoked by Endogenous Acetylcholine

As shown in chapters 3 and 4, cholinergic afferent stimulation that was sub-threshold for activating the $EPSP_M$ caused a reduction in SFA in response to a depolarizing current step delivered 2 s after pathway stimulation (n = 12; See Fig. 6.4*A* and 3.10*B*). The next series of experiments were carried out to examine whether $GABA_B$ receptor activation also affected this kind of cholinergic synaptic response as this provides information as to whether $GABA_B$ receptors act pre- or post-synaptically to modify cholinergic synaptic transmission. (-)-Baclofen (20 μM) occluded the $IPSP_B$s evoked by pathway stimulation (Fig. 6.4*A* & *B*) but only partly inhibited the reduction in SFA evoked by cholinergic afferent stimulation (n = 5; Fig. 6.4*B*). The inhibition of the reduction in SFA by (-)-baclofen (20 μM) was partially reversed by subsequent application of CGP 55845A (1 μM; n = 3; Fig. 6.4*C*). The stimulation-evoked reduction in SFA was abolished by subsequent application of atropine (1 μM; see Fig. 3.11*B*).

6.3. DISCUSSION

6.3.1. $GABA_B$ Receptor Activation Inhibits Cholinergic Synaptic Transmission

Evidence presented in this chapter suggests that there is a GABA receptor-mediated inhibition of cholinergic synaptic responses in area CA1 of the rat hippocampus. The action of both the selective $GABA_B$ receptor agonist (-)-baclofen and antagonist CGP 55845A suggests that this effect is mediated by $GABA_B$ receptors. In addition, $GABA_A$ receptor contribution to this inhibition can be excluded because $GABA_A$ receptor-mediated responses were inhibited using the selective $GABA_A$ receptor antagonist picrotoxin. Nevertheless, it is possible that $GABA_A$ receptors may

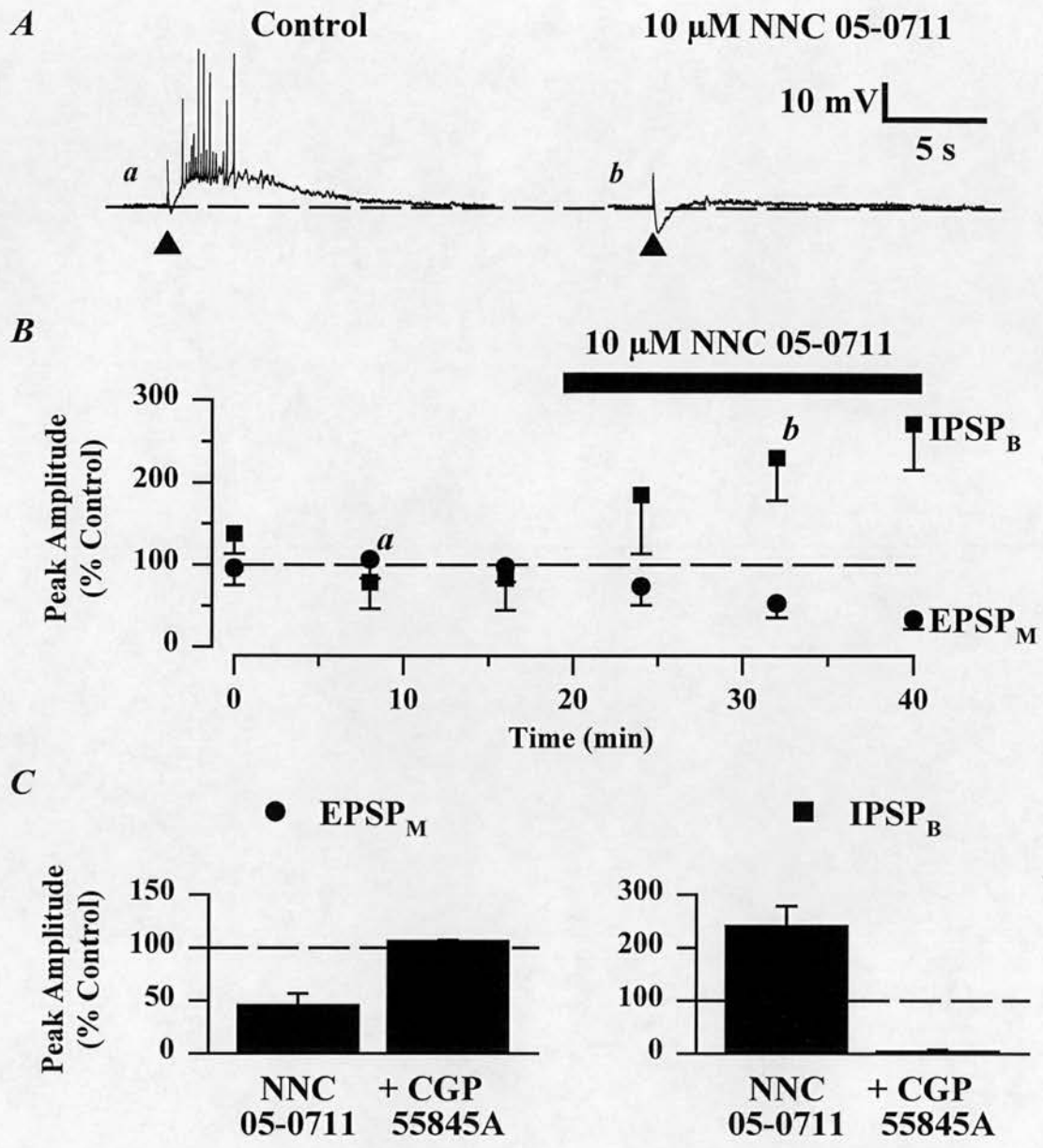

Figure 6.3. The effects of an inhibitor of GABA uptake on EPSP$_M$s and IPSP$_B$s.

In *A*, synaptic traces are representative IPSP$_B$/EPSP$_M$s recorded in control (*a*) and in the presence of NNC 05-0711 (10 μM) (*b*). The membrane potential of this neurone was -64 mV. In *B*, the graph is similar to that illustrated in Fig. 6.2*B* and plots the mean peak amplitude of successive EPSP$_M$s and IPSP$_B$s, for pooled data ($n = 4$), *versus* time to illustrate the depression of the EPSP$_M$ and the enhancement of the IPSP$_B$ by NNC 05-0711. The bars above the graph indicated the duration for which the two drugs were applied. *C*, is a bar graph illustrating pooled data for the effects of 10 μM NNC 05-0711 ($n = 4$) and 10 μM NNC 05-0711 + 1 μM CGP 55845A ($n = 2$) on the EPSP$_M$. The values plotted were calculated as described in Figure 6.1*B*. Note that NNC 05-0711 depressed the EPSP$_M$ and that CGP 55845A reversed this effect.

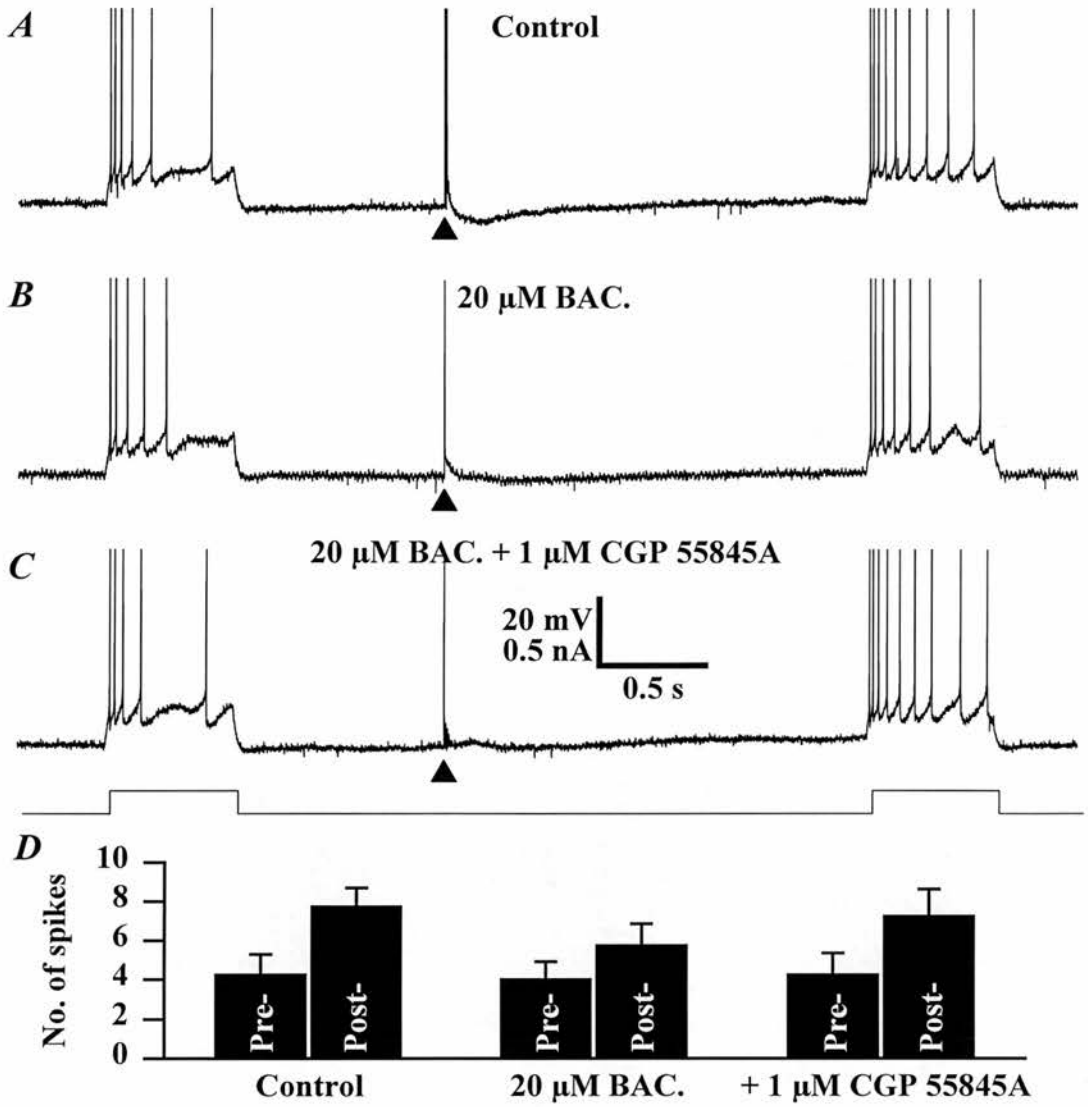

Figure 6.4. The effect of CGP 55845A on the (-)-baclofen-induced inhibition of stimulation-evoked reduction in spike frequency.

In *A–C*, traces are generated as described in Figure 4.5*A*. The responses were obtained in medium containing ionotropic glutamate and $GABA_A$ receptor antagonists (*A*), in the additional presence of 20 μM (-)-baclofen (*B*) and in the additional combined presence of 20 μM (-)-baclofen and 1 μM CGP 55845A (*C*). Note that in control medium pathway stimulation evoked an $IPSP_B$ and caused a reduction in SFA. (-)-Baclofen inhibited but did not abolish the stimulation evoked reduction in SFA and occluded the $IPSP_B$ in a CGP 55845A-sensitive manner. *D*, shows pooled data for the number of action potentials fired during a depolarizing step 1.0 s before (Pre) and 2.0 s after (Post) a sub-threshold stimulus in the absence and presence of 20 μM (-)-baclofen ($n = 5$) and in the additional presence of 1 μM CGP 55845A ($n = 5$). Note that, in the absence of a stimulation, the level of SFA evoked by the first step was not significantly different from that evoked by the second step (See Fig. 3.10). The initial membrane potential of the neurone was -65 mV.

provide an additional inhibitory influence on cholinergic synaptic transmission although further experimentation would be required to address this. Likewise, the involvement of $GABA_C$ receptors can be ruled out as

i) $GABA_C$ receptor-mediated effects are thought to be insensitive to (-)-baclofen (Bormann & Feigenspan, 1995),

ii) $GABA_C$ receptor-like effects have only been observed in hippocampal CA3 neurones in very young rats, and

iii) these effects are blocked by picrotoxin (Strata & Cherubini, 1994).

The evidence for a $GABA_B$ receptor-mediated modulation of stimulation induced reduction in SFA is less clear. (-)-Baclofen inhibits the reduction in SFA caused by pathway stimulation but does not completely inhibit this response. This is true, even at a concentration of 20 μM, which is maximal for activating postsynaptic $GABA_B$ receptors as well as inhibiting glutamate and GABA release (Thompson & Gähwiler, 1992). That said, CGP 55845A reverses this inhibition, suggesting that the inhibitory effect of (-)-baclofen is mediated by $GABA_B$ receptors. However, it remains unclear as to whether

i) 20 μM (-)-baclofen does not activate sufficient numbers of $GABA_B$ receptors to inhibit the stimulation-induced reduction of SFA, or

ii) it is not possible to inhibit ACh release by activation of $GABA_B$ receptors to the point when ACh release is sufficiently low to prevent the reduction in SFA.

It should be noted that the mechanism underlying the $EPSP_M$ requires the presence of a higher concentration of ACh than the mechanism underlying the inhibition of SFA. In addition, it is not yet clear whether these effects require release from just a single or many nerve terminals simultaneously. In this respect the possibility must be considered that only certain cholinergic fibers express $GABA_B$ receptors.

These results contrast with the A_1R-mediated inhibition of cholinergic synaptic responses (see Chapter 4). Adenosine receptor activation by 5 μM CADO substantially or completely inhibited both $EPSP_M$s and stimulation evoked reduction of SFA (see Fig. 4.7). (-)-Baclofen is, if anything, generally more potent than CADO

in its actions throughout the CNS. Thus, in terms of the postsynaptic hyperpolarization produced, (-)-baclofen has an EC_{50} of 1.1 μM (Inoue *et al.* 1985) compared with an EC_{50} of 3 μM for the effect of CADO. As such, 20 μM (-)-baclofen is a comparatively higher concentration than 5 μM CADO, and therefore would be expected to be maximal or near maximal for the depression of the $EPSP_M$. Further experiments are required to establish the precise maximal effective concentration of (-)-baclofen. Nevertheless, the results to date might be explained by

i) differences in the efficiency of receptor-effector coupling for A_1Rs and $GABA_B$ receptors

ii) differences in the transduction mechanisms which mediate the inhibitory effects of A_1Rs and $GABA_B$ on respective mAChR-mediated responses.

A more detailed quantitative analysis of the concentration-response relationships for the effects of (-)-baclofen on the $EPSP_M$ and inhibition of SFA would help to clarify whether (-)-baclofen is indeed having a differential effect on these two mAChR-mediated responses. These experiments would also serve to suggest whether (-)-baclofen is acting pre- or post-synaptically to inhibit cholinergic synaptic responses.

To date, evidence for $GABA_B$ receptor-mediated inhibition of ACh release in the CNS is limited, providing some support for a postsynaptic site of action of (-)-baclofen (Waldmeier & Baumann, 1990). In one study however, $GABA_B$ receptor activation has been shown to inhibit the release of ACh in the superior colliculus, although the pharmacological profile of that receptor did not match that of a classical $GABA_B$ receptor (Wichmann *et al.* 1987). Alternatively, (-)-baclofen may be acting both pre- and post-synaptically, or purely postsynaptically, to inhibit cholinergic responses. In this respect, in hippocampal pyramidal neurones, postsynaptic activation of $GABA_B$ receptors (like A_1R activation) evokes a hyperpolarization which is accompanied by a reduction in input resistance, mediated by an inwardly rectifying K^+ conductance (Newberry & Nicoll, 1984b; Blaxter & Carlen, 1985; Inoue *et al.* 1985). Nevertheless, although (-)-baclofen depressed $EPSP_M$s at a concentration which also caused a substantial hyperpolarization, this hyperpolarization alone cannot account for the depression, as this was routinely

compensated for using positive current injection. Equally, the reduction in input resistance caused by activation of a K^+ conductance could feasibly limit the extent of depolarization during the $EPSP_M$ by shunting of membrane currents. This possibility, however, is unlikely in the case of the effect of NNC 05-0711, as the maximum duration of the $IPSP_B$, even in the presence of NNC 05-0711, is little greater than 1 s, which is more than 1 s less than the time to peak of the $EPSP_M$. Likewise, the facilitatory effect of CGP 55845A alone on the $EPSP_M$ cannot be explained by the shunting of membrane currents, unless shunting in the first 1 s of the $EPSP_M$ affects its activation.

Finally, it is possible that $GABA_B$ receptor activation somehow interferes with the postsynaptic signal transduction of mAChR-mediated responses. As mentioned above, $GABA_B$ receptors and A_1Rs act via very similar signal transduction mechanisms. Thus, both receptors are coupled to inhibitory G-proteins of the G_i or G_o subtype (Dutar & Nicoll, 1988b; Thalmann, 1988; Trussel & Jackson, 1987) and both receptors negatively couple to the AC/cAMP system (Sattin & Rall, 1970; Wojcik *et al.* 1990). However, as discussed in chapter 5, it is unlikely that these mechanisms, when operating postsynaptically, are responsible for the depression of cholinergic responses because M_1 and M_3 mAChRs couple to the G_q/G_{11} subtypes of G-proteins which couple to the PLC second messenger system. Nevertheless, $GABA_B$ receptor activation can enhance IP_3 production (Karbon *et al.* 1990; Wojcik *et al.* 1990; Crawford & Young, 1990) which conceivably might interfere with the M_1/M_3 mAChRs mediated effects.

The true mechanism of $GABA_B$ receptor mediated inhibition of mAChR-mediated effects cannot be elucidated until it is clear

i) whether the effect of (-)-baclofen is pre- or postsynaptic, and

ii) which mAChRs and transduction mechanisms mediate $EPSP_M$s and inhibition in SFA.

6.3.2. ENDOGENOUS GABA ACTIVATES $GABA_B$ RECEPTORS TO INHIBIT CHOLINERGIC SYNAPTIC RESPONSES

It is already well known that GABA, which is synaptically released from hippocampal interneurones, has a physiological role in the hippocampus. In this respect, synaptically released GABA activates both $GABA_A$ and $GABA_B$ receptors that mediate the early $IPSP_A$ and late $IPSP_B$, respectively (Krnjevic & Schwartz, 1967; Dutar & Nicoll, 1988c). In addition, synaptically released GABA can activate presynaptic $GABA_B$ auto- and hetero-receptors to inhibit GABA- and glutamate-mediated synaptic transmission, respectively (Dutar & Nicoll, 1988b; Harrison *et al.* 1988). The possibility that endogenous GABA could also inhibit cholinergic synaptic transmission was suggested here by the action of the selective $GABA_B$ receptor antagonist CGP 55845A to increase the amplitude of $EPSP_M$s beyond control levels. This observation implies that endogenous GABA might inhibit $EPSP_M$s under control conditions. This suggestion is further strengthened by the effects of NNC 05-0711, which has been shown to selectively inhibit the uptake of GABA from synaptosomal and neuronal preparations (Suzdak *et al.* 1992). In this respect, NNC 05-0711 increased the amplitude of $IPSP_B$s and inhibited $EPSP_M$s in a CGP 55845A-sensitive manner. However, it is unclear whether the GABA, that activates the $GABA_B$ receptors that inhibit the $EPSP_M$, is actually present in the extracellular medium or whether it is released by, for example, GABAergic interneurones when afferent stimulation is delivered. An intriguing possibility is that GABA is co-released from cholinergic terminals or from septohippocampal GABAergic projections, instead of intrinsic GABAergic interneurone terminals.

6.4. SUMMARY

There is a $GABA_B$ receptor-mediated inhibition of cholinergic synaptic responses in area CA1 of the rat hippocampus. Endogenous GABA may activate these $GABA_B$ receptors to inhibit $EPSP_M$s. It is unclear whether this effect is mediated pre- or post-synaptically. However, the mechanism of this depressant action appears to differ from that of the presynaptic adenosine receptor-mediated inhibition of cholinergic synaptic responses.

CHAPTER 7

GENERAL CONCLUSIONS

7.1. CHOLINERGIC SYNAPTIC TRANSMISSION IN THE HIPPOCAMPUS

The initial aim of this thesis was to establish a protocol for the investigation of cholinergic synaptic responses at rat septohippocampal CA1 synapses in isolation. Like earlier studies, mAChR-mediated synaptic responses were evoked using stimulation of the septohippocampal pathway in *s. oriens* (Cole & Nicoll, 1983). For the first time, however, $EPSP_M$s were evoked using just a single stimulus. In this respect, $EPSP_M$s evoked in the present study differed from those evoked previously in that:

i) no activity dependent changes, e.g. facilitation/depression, were associated with their activation.

ii) they were routinely evoked in the absence of cholinesterase inhibitors that inhibit the breakdown of ACh.

iii) they were isolated from ionotropic glutamate and GABA receptor mediated synaptic responses to avoid possible effects on the induction and expression of $EPSP_M$s (such as the $GABA_B$ receptor-mediated inhibition described in chapter 6).

iv) they were shown, for the first time, to be reproducible over a prolonged period (several hours).

v) they were reproducibly evoked every 5–10 minutes (compared to every 15 minutes, as in previous studies).

This experimental protocol was utilized for the investigation of cholinergic synaptic transmission and, in particular, to study the modulation of mAChR-mediated synaptic responses by adenosine and GABA receptor activation.

7.1.1 Cholinergic Synaptic Responses

Data presented in this study have shown that synaptic activation of mAChRs on hippocampal CA1 pyramidal neurones, using the protocol described above, inhibits spike frequency adaptation (SFA) and causes a slow depolarization or $EPSP_M$. In addition to activation of these classic mAChR-mediated responses, synaptic activation of mAChRs should, in theory, mimic other effects of mAChR agonists. In this respect, some preliminary data has shown that synaptic activation of mAChRs may affect the duration of action potentials and evoke membrane oscillations in the frequency range of theta rhythm (see Chapter 3). As such, this study extends the mechanisms by which synaptic activation of mAChRs can modulate postsynaptic excitability in the hippocampus adding to the many mechanisms described over the past two decades (Dodd *et al.* 1981; Cole & Nicoll, 1983; Figenschou *et al.* 1996).

Both cholinergic systems and the hippocampus are thought to be closely involved in the process of learning and memory formation (Decker & McGaugh, 1991; Aigner, 1995). Much evidence for this comes from behavioural experiments which show that disruption of the cholinergic system, using either mAChR antagonists or lesions of the septohippocampal pathway, impairs the ability of rats and humans to perform learning and memory tasks (Bartrus *et al.* 1982). As such, the increased excitability afforded by mAChRs may be important for modulating the effects of other neurotransmitters, e.g. glutamate, in the hippocampal region. In this respect, at a synaptic level, it has been shown that activation of M_1 mAChRs can facilitate the induction of LTP, a synaptic modification believed to be important in learning (Blitzer *et al.* 1990).

7.1.2 Modulation of Cholinergic Synaptic Responses

In view of the potential importance of mAChR-mediated effects in cognitive function, mechanisms which control their activation are likely to provide a further level of control of learning and memory processes. Whilst previous studies have shown that ACh turnover *in vivo*, or evoked ACh release from isolated hippocampal slices, is inhibited by A_1R activation (Cunha *et al.* 1994), I have demonstrated, for the first time, the functional consequences of this inhibition. This is described in

terms of the presynaptic action of A_1Rs to inhibit the effects of synaptic activation of mAChRs (chapter 4 & 5).

In addition to A_1R-mediated modulation, this thesis also dealt with a modulation of cholinergic synaptic transmission by $GABA_B$ receptor activation. A_1Rs and $GABA_B$ receptors share many similarities, in that

i) both positively couple to K^+ and negatively couple to Ca^{++} channels in the hippocampus,

ii) both are G-protein coupled receptors and negatively couple to the AC/cAMP system, and

iii) both are located pre- and post-synaptically in the hippocampus.

However, GABA and adenosine differ fundamentally in that, while GABA is released synaptically at specific locations and acts predominantly at that site of release, adenosine is produced and released non-synaptically from many locations and may act at sites distant from the point of release. In this respect, while mechanisms exist for the breakdown and the uptake of adenosine, adenosine is thought to maintain a basal extracellular concentration and exert a tonic action in neuronal tissue. In contrast, GABA has a more temporally discrete action. This difference may affect the function of these modulators, as GABA will tend to act predominantly at points close to where GABA synapses are active, whereas adenosine could act in a "blanket" manner to inhibit numerous cholinergic inputs at the one time. As such, the two neuromodulatory systems are likely to modulate mAChR synaptic transmission to different extents during different patterns of neuronal activity, whether this be during normal physiological functioning or in pathophysiological states.

7.2. FUTURE RESEARCH

7.2.1 CHOLINERGIC SYNAPTIC TRANSMISSION IN THE HIPPOCAMPUS

Although this study has concentrated on the modulation of cholinergic synaptic responses by other receptor systems, there is much to be learned about the pharmacology of mAChR-mediated responses and the transduction mechanisms that

mediate these responses. Indeed, in addition to the large variety of conductances which are known to be modulated by mAChRs, there are a number of effects (e.g. on action potential kinetics) which are poorly characterized. Further analysis of the variety of conductances inhibited or activated by synaptic activation of mAChRs, using voltage clamp techniques and using inhibitors of a variety of transduction mechanisms (e.g. inhibition of PKC using dihydrosphingosine) would help to clarify the mechanisms of mAChR activation in the hippocampus. If drugs which selectively modulate the cholinergic system are to be effective with few side effects, it will also be necessary to elucidate which subtype(s) of mAChR mediate each of these effects and to develop more subtype selective mAChR agonists and antagonists.

Another factor which limits the ease of study of cholinergic synaptic transmission in the hippocampus, is the fatigue of $EPSP_M$s on repetitively stimulating at interstimulus intervals of less than 5-8 min. Initially, further work might focus on determining whether the mechanism behind this process is pre- or post-synaptically mediated. A clue to the mechanism underlying this depression may come from the hyperpolarization sometimes observed following an $EPSP_M$ as this often lasts over the time period during which the fatigue occurs. The prolonged duration of the fatigue might suggest effects on the postsynaptic biochemistry of the cholinergic signaling pathway. A G-protein dependent desensitization of mAChR-mediated responses has been reported in the hippocampus (Guérineau *et al.* 1997). As such, it would be interesting to study the involvement of G-proteins in this fatigue. However, compounds which affect G-protein function, such as the G-protein activator and inhibitor GTP-γ-S and GDP-β-S, respectively, may well disable the $EPSP_M$ itself. In addition, whether mAChRs themselves or other receptor systems, (e.g. galanin, which is coreleased with ACh), mediate the hyperpolarizing response should be addressed. Indeed, the mechanism(s) of this response is/are also unclear and therefore may involve

i) purely ionic mechanisms or

ii) activation of intracellular signal transduction processes such as the PLC/IP_3

system.

The mechanism underlying the fatigue of $EPSP_M$s may have important functional consequences for cholinergic function *in vivo*. Depression of $EPSP_M$s or a prolonged hyperpolarization, could limit the effectiveness of the cholinergic system to modulate mnemonic processing and, more importantly, could limit the effectiveness of cholinergic drug treatment in, for example, Alzheimer's Disease.

The effects of cholinergic activity in the hippocampus on the induction of long term synaptic plasticity should also be addressed. This possibility may be investigated by examining the effect of cholinergic synaptic activation on synaptic plasticity in area CA1 and CA3 of the rat hippocampus. The success of such a study will depend on the degree of synapse specificity of the modulatory effects of cholinergic synaptic activation. It may be that only synapses, subject to plastic change, which are spatially adjacent to cholinergic synapses will be affected at lower levels of cholinergic stimulation. A further complicating factor may be the multiple effects of mAChR activation in the hippocampus. In this respect, higher levels of cholinergic synaptic activation could lead to a depression rather than potentiation of the glutamatergic synaptic responses.

$EPSP_M$s have not been shown to occur during normal brain activity *in vivo*. Indeed, such prolonged excitation would be expected to be detrimental to hippocampal neurones. As such, it may be that these large, prolonged depolarizations represent a pathophysiological response relevant, for example, to when hippocampal inhibition is impaired. Indeed, the presence of several mechanisms for the control of such depolarizing responses (the effects of adenosine and GABA and the depression of responses on repeated stimulation) suggests a need for control to prevent the overactivation of the cholinergic system. It would be interesting if, using simultaneous paired cell recording from neurones in the septum and hippocampus, it is possible to activate $EPSP_M$s purely by depolarizing a presynaptic septal cholinergic neurone to fire just a single action potential or a series of action potentials. However, a simultaneous, synchronized stimulation of a number of septal cholinergic neurones may be required to evoke even small $EPSP_M$s. As such, it is possible that cholinergic

synaptic function *in vivo* consists of only small depolarizations at single synapses and/or alterations in the firing frequency of pyramidal neurones. In this respect, mAChR activation would maintain a modulatory influence on excitability of neurones and possibly individual synapses, rather than having a direct excitatory action. Nevertheless such a modulatory influence would have profound effects on the functioning of neuronal networks and synaptic integration.

7.2.2 Adenosine Receptor Modulation of Cholinergic Synaptic Transmission

The present study shows that A_1R activation is an effective mechanism for the control of both depolarizing responses and inhibition of SFA. Indeed, results presented in chapter 5 suggest that adenosine, endogenous to the hippocampus, may tonically modulate cholinergic responses *in vitro* at least. It is possible that *in vivo*, basal levels of adenosine tonically modulate mAChR-mediated synaptic transmission. Some studies have estimated the concentration of endogenous adenosine to be 0.03-2.0 μM in brain tissue (Zetterström *et al.* 1982, Fredholm *et al.* 1984). In the present study adenosine was effective only at much higher concentrations. However, this does not equate to the concentration of adenosine at cholinergic synapses in the slice preparation due to diffusional constraints and uptake mechanisms that are active within the slice. In this respect, where adenosine is released there may be no uptake such that adenosine levels may be particularly high. Nevertheless, an increased tonic level of adenosine may be required to inhibit cholinergic responses *in vivo*.

Adenosine is released from neuronal tissue and may exert control over of a number of transmitter systems, although the source of this adenosine is controversial. One source of adenosine is from the breakdown of ATP. Hippocampal cholinergic nerve terminals possess the enzymatic machinery to metabolize extracellular ATP to adenosine (Cunha *et al.* 1992). ATP is stored and co-released with ACh at cholinergic motor nerve terminals (Downall *et al.* 1974; Silinsky, 1975) where both ATP and adenosine inhibit evoked and spontaneous release of ACh (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1973). There is no evidence of synaptic release of

ATP in the hippocampus, although metabolic ATP may be released in the hippocampus during metabolic stress, e.g. during hypoxia. As such, adenosine is thought to control excitability during hypoxia. A natural extension of the present study, therefore, would be to address the effects of experimentally induced hypoxia on cholinergic synaptic responses and the role of adenosine in the control of cholinergic excitation during hypoxia.

Whilst this study deals with the presynaptic aspects of control, it is, however, still possible that A_1Rs affect mAChR-mediated responses postsynaptically as well as presynaptically. In this respect, an inhibitory postsynaptic effect of adenosine may be "swamped" by the large effects of mAChR activation. To further investigate this possibility, it may be prudent to use a lower level of mAChR activation in order to increase the chance and observing any the postsynaptic inhibitory actions of adenosine. As such, a better technique for the investigation of lower levels of ACh release would be to employ patch clamp techniques to voltage clamp neurones and provide a more accurate quantification of mAChR-mediated inhibition of I_{AHP}, for example. This would allow the investigation of more subtle effects of adenosine receptor activation on a lower level of mAChR activation which is required to produce this postsynaptic mAChR-mediated effect. One limitation of this method, however, is the potential to dialyze intracellular contents essential for the transduction of the mAChR-mediated response. This may be overcome by the use of the nystatin perforated patch approach, as this will limit the extent of the dialysis.

Adenosine receptor activation has been shown to bidirectionally modulate the release of ACh in area CA3 but not area CA1 of the hippocampus (Cunha *et al.* 1994). In this respect, in area CA3, activation of A_1Rs inhibits ACh release and A_{2a}Rs enhances ACh release. Thus, adenosine is able to differentially affect cholinergic synaptic transmission in different areas of the same structure. Further studies might concentrate on the modulation of cholinergic synaptic transmission at CA3 pyramidal neurones and look for possible interactions between A_1R and A_{2a}R-mediated effects. Depending on the location of these receptors and the concentration of adenosine to which they are exposed, differential effects on $EPSP_M$s could be observed.

Preliminary results presented in chapter 5 suggest that the A_1R-mediated inhibition of $EPSP_M$s may in part involve 4-AP-sensitive K^+ channels and inhibition of cAMP turnover. Further work might focus on more detailed classification of any K^+ channels involved in the inhibitory action of adenosine using the K^+ channel antagonist α-dendrodotoxin, for example. In addition, the role of the AC/cAMP system should be addressed using, for example the AC activator forskolin and inhibitors such as SQ 22,536.

Based of the concept that presynaptic A_1Rs modulate cholinergic synaptic transmission, the involvement of Ca^{++} channels in the A_1R-mediated modulation of cholinergic synaptic responses should also be addressed. In studies of A_1R-mediated inhibition of glutamate release, A_1R activation appears to involve the inhibition of N- and possibly Q-type voltage activated Ca^{++} channels (Wu & Saggau, 1994). It would be advantageous, firstly, to ascertain which subtype(s) of voltage activated Ca^{++} channels modulate the release of ACh *per se* in the hippocampus using ω-conotoxin-GVIA and ω-agatoxin-IVA. The action of A_1R agonists on the responses which remain following application of individual or combinations of Ca^{++} channel blockers could then give an indication as to the subtype(s) that may be involved in the A_1R-mediated inhibition.

7.2.3 GABA Receptor Modulation of Cholinergic Synaptic Transmission

Less well defined than the effects of A_1R activation on mAChR-mediated responses are the effects of $GABA_B$ receptor activation. It is unclear why (-)-baclofen, even at a high concentration, did not completely inhibit the mAChR-mediated inhibition of SFA. The effect of a "supramaximal" concentration of (-)-baclofen was not systematically tested on the $EPSP_M$, but at the highest concentration used this agonist did not completely inhibit the $EPSP_M$. If the effect of (-)-baclofen is presynaptic, it may be that $GABA_B$ receptor activation does not inhibit the release of ACh as effectively as A_1R activation. In this respect, there may be cholinergic fibers which differentially express $GABA_B$ receptors whereas all express A_1Rs. Such a concept is not unrealistic since differences in cholinergic inputs do exist. Thus, for example

galanin inhibits cholinergic synaptic responses in ventral but not dorsal hippocampus (Dutar *et al.* 1989). As to the source of GABA, there are two possible sources:

i) from interneurones activated by the stimulus used to evoke the $EPSP_M$, or

ii) from excitatory inputs e.g. from cholinergic afferents themselves.

The involvement of GABA released from direct activation of interneurones could be tested by evoking an $EPSP_M$ by stimulating at a point distant from the CA1, e.g. the fimbria, and applying a GABA uptake inhibitor e.g. NNC 05-0711. If there is no effect of the GABA uptake inhibitor then this would suggest that release of endogenous GABA from cholinergic afferents is not responsible for inhibition of the $EPSP_M$ and that GABA release under normal experimental conditions is as a result of direct activation of interneurones in the CA1 alone.

7.2.4 Modulation of Cholinergic Synaptic Transmission by Other Mechanisms

As well as a modulation by adenosine and $GABA_B$ receptors, previous studies together with those ongoing in this laboratory have shown that other receptor systems modulate cholinergic synaptic responses and ACh release. These include galanin receptors and mAChRs themselves (Dutar *et al.* 1989; Pepeu *et al*, 1990). mAChR agonists and antagonists have been shown to modulate the release of radiolabelled ACh. As such, it may be possible, using selective cholinergic agonists and antagonists, to promote or inhibit cholinergic synaptic responses, providing the receptors prove to be pharmacologically distinguishable from postsynaptic mAChRs. The functional significance of presynaptic mAChRs in modulating evoked $EPSP_M$s may have already been demonstrated in the observation that atropine sometimes enhanced $EPSP_M$s prior to inhibiting them (data not shown). As such, in a few cases atropine may have affected presynaptic mAChRs more rapidly than postsynaptic mAChRs, so transiently enhancing the release of ACh and hence the amplitude of $EPSP_M$s. However, more detailed experiments are required to confirm this preliminary observation.

Recent studies have shown that β-amyloid peptide fragments have an inhibitory

effect on a number of cholinergic neurotransmitter processes in neuronal cell cultures. In addition, β-amyloid peptide fragments can inhibit ACh release in hippocampal and cortical slices (see Auld *et al.* 1998 for review). β-amyloid peptide has been found to be deposited in neuritic plaques throughout the brains of patients with Alzheimer's Disease (AD). As such, investigation of the effect(s) of such peptide fragments on cholinergic synaptic transmission, using the methods described in this thesis, would allow a more detailed analysis of the inhibitory mechanism of their action. This would therefore provide a better understanding of the processes involved in the initial stages of AD.

7.3. POTENTIAL CLINICAL USES FOR MODULATORS OF CHOLINERGIC SYNAPTIC TRANSMISSION

Cholinergic neurones are known to degenerate in AD (Coyle, 1983; Fibiger, 1991). In addition, there is a large body of evidence to suggest that activation of mAChRs facilitates, whereas antagonism and lesioning of cholinergic pathways impairs learning and memory processes (Bartrus *et al.* 1982). Moreover, mAChR activation has been shown to be involved in the induction of certain forms of long-term synaptic plasticity (e.g. LTP) which are thought to represent *in vitro* models of learning and memory processes *in vivo* (Blitzer *et al.* 1990; Auerbach & Segal, 1996). As such, agents which modify cholinergic function may have clinical uses in disease processes where mnemonic processing is compromised, e.g. AD (Giacobini, 1990; Thal, 1996).

AD and dementia syndromes are often associated with aging. In this respect cholinergic function has been investigated in aged animals. Cholinergic receptor binding has been shown to be compromised in aged gerbils and rats (Hara *et al.* 1992). However, changes in mAChR binding in postmortem human brains of AD patients are less clear. In some, mAChR binding is unaffected in the hippocampus but not in the cortex of AD patients (Lang & Henke, 1983). In contrast, another group has reported an increase in mAChR number in AD patients, although this is thought to be due to a compensatory increase in receptor number in response to reduced ACh release. In the same study, however, reduced mAChR binding was

seen in more severe AD cases, although it is unclear whether this is due to a loss of select populations of neurones (Probst *et al.* 1988). In addition, functional studies have shown that ACh release and slow cholinergic synaptic responses are impaired in aged rats (Potier *et al.* 1992; Pedata *et al.* 1983b; Wu *et al.* 1988). As such, there is a large body of evidence to suggest that therapies that facilitate cholinergic function may be useful in the treatment of dementia or AD. In this respect, drug companies continue to develop subtype selective mAChR agonists as well as AChE inhibitors.

In contrast, endogenous "adenosinergic" inhibition has been shown to be enhanced in aged animals (Bauman *et al.* 1992). Based on the present evidence, this enhanced adenosinergic inhibition would contribute to the decline of cholinergic function in aged animals due to an increased A_1R-mediated inhibition of cholinergic synaptic transmission. As such, therapies that inhibit A_1R-mediated inhibition of cholinergic synaptic transmission may be advantageous in the treatment of dementia or AD. However, A_1R agonists may not be of clinical use because of their multiple actions throughout the nervous system and vasculature.

A similar problem exists with respect to the clinical use of $GABA_B$ receptor antagonists to counteract a decline in cholinergic function, in that GABA is the most prominent inhibitory neurotransmitter in the CNS. As such, drugs which affect $GABA_B$ receptor function would potentially have poor specificity and may result in, for example, epileptogenesis, due to a reduced inhibition of glutamatergic synaptic transmission in the hippocampus. Saying this, to date $GABA_B$ receptor antagonists have been shown to be reasonably well tolerated by humans and indeed are capable of enhancing cognitive performance in humans and primates (Mondadori *et al.* 1992). More promising may be the development of drugs which affect other receptor systems which inhibit or enhance cholinergic function but have fewer effects in the brain than A_1Rs and $GABA_B$ receptors. Of possible interest is the limited distribution of A_{2a}Rs. The enhancement of ACh release by adenosine has been noted in area CA3 in the hippocampus but not the CA1 (Cunha *et al.* 1994). In this respect, A_{2a}R agonists could be used to facilitate cholinergic synaptic function although again these receptors also promote glutamate release (Okada *et al.* 1992). In addition to

modulation of cholinergic function by such heteroreceptors, mAChR autoreceptors may prove to be pharmacologically distinct from mAChRs which mediate postsynaptic responses. As such, selective enhancement of ACh release could be achieved using antagonists at these receptors. Clearly there are numerous possibilities for modulation of cholinergic synaptic transmission, however, it remains to be seen whether any of these has potential for therapeutic usage.

In this respect, the development of cholinergic therapies in the past has led to only limited success in relieving the cognitive impairment found in AD and dementia (Thal, 1996). Part of the problem no doubt reflects the fact that cholinergic impairment is thought to represent just one aspect of the cognitive impairments found in such disorders. Nevertheless, other disease processes, e.g. Parkinson's disease and Huntingdon's disease, also exhibit a degree of cholinergic dysfunction and might benefit more from the development of drugs which modify cholinergic function *in vivo*.

Finally an additional problem, with respect to the development of cholinergic therapies, exists. Systemic administration of drugs which act at AChRs will result in many side effects due to the many peripheral effects mediated by AChRs both in sympathetic and parasympathetic nervous system. This has been a long standing problem with many CNS drugs such as neuroleptics, which classically exhibit atropine-like side effects, such as dry mouth and constipation. Such problems can be counteracted to some extent by developing pro-drugs which can be converted to the active form at or near the site of action.

Whatever the case the development of therapies which modulate the cholinergic system in the CNS will require further investigation of the pharmacology and mechanisms of cholinergic synaptic transmission and its possible functional significance in mnemonic processing.

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9. APPENDIX

9.1 PUBLICATIONS

R.A. Morton, D.O. Bulters & C.H. Davies (1997). $GABA_B$ receptor-mediated modulation of muscarinic acetylcholine receptor-mediated synaptic responses in the rat hippocampus. *British Journal of Pharmacology* **122,** 274P.

R.A. Morton & C.H. Davies (1997). Regulation of muscarinic acetylcholine receptor-mediated EPSPs by adenosine receptors in the rat hippocampus. *Journal of Physiology* **502(1),** 75-90.

R.A. Morton & C.H. Davies (1996). Adenosine A_1 receptors presynaptically inhibit muscarinic cholinergic EPSPs in the rat hippocampus. *Society for Neuroscience, Abstracts*, **22 (3),** 779.10.

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Adenosine receptor-mediated modulation of cholinergic synaptic transmission in the rat hippocampus *in vitro*

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The hippocampus receives a dense cholinergic input from the medial septal nucleus, which is believed to play an important role in learning and memory processes (Dutar *et al.* 1995). Stimulation of this pathway has been shown to evoke a slow muscarinic cholinergic receptor (mAChR)-mediated excitatory postsynaptic potential (EPSP; Cole & Nicoll, 1984). Unlike amino acid-mediated synaptic transmission, this synaptic input has received relatively little attention in terms of its modulation by other receptor systems. The purpose of the present study, therefore, was to investigate how adenosine receptor activation affects isolated slow cholinergic EPSPs.

Transverse hippocampal slices from 2- to 4-week-old female Wistar rats were prepared using standard techniques and maintained at 33–35 °C in an interface recording chamber. Intracellular recordings were made from CA1 pyramidal neurones using electrodes (70–110 MΩ) filled with potassium methylsulphate (2 M). In all experiments 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μM), D-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid (CGP 40116; 50 μM), picrotoxin (50 μM) and 3-*N*-(1-(S)-(3,4-dichlorophenyl) ethyl) amino-2-(*S*)-hydroxypropyl-*p*-benzyl-phosphonic acid (CGP 55845A; 1 μM) were included in the perfusing medium to block α-amino-3-hydroxy-5-methyl-1,4-isoxazole (AMPA), *N*-methyl-D-aspartate (NMDA), γ-aminobutyric acid $(GABA)_A$ and $GABA_B$ receptors, respectively. Under these conditions, reproducible slow EPSPs could be evoked every 8 min using a single, high intensity stimulus delivered in *stratum oriens*. The amplitude and duration of these EPSPs were increased by physostigmine (1 μM), and were inhibited by atropine (1–2 μM).

2-Chloroadenosine (CADO, 0·2–2 μM) reversibly depressed slow EPSPs in a concentration-dependent manner; for example, at 1 μM, CADO depressed the EPSP by 87 ± 9% ($n = 8$, mean ± S.E.M.). Likewise, 2-chloro-N^6-cyclopentyl adenosine (CCPA, 0·1–0·4 μM) inhibited slow EPSPs ($n = 3$), whereas 2-*p*-(2-carboxyethyl) phenethylamino-5′-*N*-ethyl-carboxamidoadenosine (CGS 21680, 1 μM) did not ($n = 3$). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX; 200 nM) fully reversed the CADO-induced depression of slow EPSPs such that responses in DPCPX plus CADO (1 μM) were 107 ± 11% of control ($n = 3$).

These data suggest that in the presence of ionotropic amino acid receptor antagonists it is possible to evoke slow mAChR-mediated EPSPs in CA1 pyramidal neurones that are depressed by adenosine A_1 receptor activation.

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779.10

Adenosine A_1 Receptors Presynaptically Inhibit Muscarinic Cholinergic EPSPs in the Rat Hippocampus. R.A. Morton and C.H. Davies. (SPON: Brain Research Association) Department of Pharmacology, University of Edinburgh, 1 George Square, Edinburgh, EH8 9JZ, UK.

Stimulation of septo-hippocampal cholinergic fibers in *stratum oriens* produces a slow muscarinic cholinergic excitatory post-synaptic potential (EPSP) in CA1 pyramidal neurones of the rat hippocampus. This pathway is believed to be particularly important in learning and memory in the vertebrate central nervous system. As such, mechanisms that control this synaptic input are of particular interest. In this respect, we have demonstrated previously that adenosine A_1 receptors inhibit muscarinic EPSPs in CA1 pyramidal neurones (Morton & Davies, *J. Physiol.*, 1996). Here we focus on whether this effect is pre- or post-synaptically mediated.

Extracellular recording from *stratum radiatum* and intracellular current clamp recording from CA1 pyramidal neurones was used to compare known pre- and post-synaptic effects of the adenosine receptor agonist 2-chloroadenosine (CADO) with its effect on muscarinic EPSPs. CADO caused a concentration dependent presynaptic inhibition of field recorded glutamate EPSPs (n=4) with an IC_{50} of 0.6 μM. Postsynaptically, CADO caused a hyperpolarization (up to 15 mV) and a concomitant reduction in input resistance (n=4) with an EC_{50} of 2.7 μM. The IC_{50} for the depressant action of CADO on cholinergic EPSPs was 0.4 μM, which is comparable with the IC_{50} for the presynaptic inhibition of glutamatergic EPSPs. All these effects were reversed by DPCPX (200 nM). In a separate series of experiments, brief bath applications of carbachol caused consistent depolarizations and increases in input resistance. These effects were unaffected by CADO (1 μM; n=4) but were abolished by atropine (1-5 μM; n=3).

These data suggest that CADO exerts its inhibitory effect on muscarinic cholinergic synaptic transmission by inhibiting acetylcholine release via presynaptic adenosine A_1 receptors. (R. Morton is an M.R.C. funded Ph.D. student).

274P $GABA_B$ RECEPTOR-MEDIATED MODULATION OF MUSCARINIC ACETYLCHOLINE RECEPTOR-MEDIATED SYNAPTIC RESPONSES IN THE RAT HIPPOCAMPUS

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The hippocampus receives a dense cholinergic input from the medial septal nucleus, which is believed to have an important role in learning and memory processes *in vivo*. Stimulation of this pathway *in vitro* has been shown to evoke muscarinic acetylcholine receptor (mAChR)-mediated responses in hippocampal pyramidal neurones. Recently we have shown that adenosine A_1 receptors, which couple to similar effector systems to γ-aminobutyric acid $(GABA)_B$ receptors, modulate mAChR-mediated responses (Morton & Davies, 1997). Here we report the effects of $GABA_B$ receptor activation on mAChR-mediated synaptic responses in the CA1 region of the rat hippocampus.

Transverse hippocampal slices from 2- to 4-week-old female Cob-Wistar rats were prepared using standard techniques and maintained at 32 ± 2 °C in an interface recording chamber continuously perfused with artificial cerebrospinal fluid gassed with 95 % O_2/5 % CO_2. Intracellular current clamp recordings were made from CA1 pyramidal neurones using electrodes (70-110 MΩ) filled with potassium methylsulphate (2 M). In all experiments 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX; 2-4 μM), D-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid (CGP 40116; 50 μM) and picrotoxin (50 μM) were included in the perfusing medium to block α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and $GABA_A$ receptors respectively. Under these conditions, reproducible $GABA_B$ receptor-mediated IPSPs ($IPSP_BS$) followed by slow mAChR-mediated EPSPs ($EPSP_MS$) could be evoked using a single stimulus delivered in the *stratum oriens*.

The selective $GABA_B$ receptor agonist baclofen (5 μM) occluded $IPSP_BS$ and inhibited $EPSP_MS$ by 71 ± 15 % (n = 4, mean ± s.e.mean). The selective $GABA_B$ receptor antagonist [1-(*S*)-3,4-(dichlorophenyl)ethyl] amino-2-(*S*)-hydroxypropyl-*p*-benzyl-phosphonic acid (CGP 55845A; 1 μM), when applied alone, inhibited $IPSP_BS$ and increased the amplitude of $EPSP_MS$ to 253 ± 74 % of control responses (n = 3). In contrast, the GABA uptake inhibitor diphenylmethanone, 0-[2-(3-carboxy-1,2,5,6-tetrahydro-1-pyridinyl)ethyl]oxime, HCl (NNC 05-0711, 10 μM) increased the amplitude of $IPSP_BS$ to 241 ± 38 % of control amplitudes and depressed $EPSP_MS$ by 58 ± 10 % (n = 4). CGP 55845A (1 μM) reversed this inhibition of $EPSP_MS$ and abolished the $IPSP_BS$ (n = 2).

These data suggest that there is a $GABA_B$ receptor-mediated inhibition of cholinergic synaptic transmission which can be activated by endogenously released GABA.

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Regulation of muscarinic acetylcholine receptor-mediated synaptic responses by adenosine receptors in the rat hippocampus

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1. Intracellular current clamp recordings were made from CA1 pyramidal neurones in rat hippocampal slices. Experiments were performed in the presence of ionotropic glutamate receptor antagonists and γ-aminobutyric acid (GABA) receptor antagonists to block all fast excitatory and inhibitory synaptic transmission. A single stimulus, delivered extracellularly in the stratum oriens, caused a reduction in spike frequency adaptation in response to a depolarizing current step delivered 2 s after the stimulus. A 2- to 10-fold increase in stimulus intensity evoked a slow excitatory postsynaptic potential (EPSP) which was associated with a small increase in input resistance. The peak amplitude of the EPSP occurred approximately 2·5 s after the stimulus and its magnitude (up to 30 mV) and duration (10–50 s) increased with increasing stimulus intensity.

2. The slow EPSP was unaffected by the metabotropic glutamate receptor antagonist (+)-α-methyl-4-carboxyphenylglycine ((+)-MCPG; 1000 μM) but was greatly enhanced by the acetylcholinesterase inhibitor physostigmine (1–5 μM). Both the slow EPSP and the stimulus-evoked reduction in spike frequency adaptation were inhibited by the muscarinic acetylcholine receptor (mAChR) antagonist atropine (1–5 μM). These results are consistent with these effects being mediated by mAChRs.

3. Both the mAChR-mediated EPSP ($EPSP_m$) and the associated reduction in spike frequency adaptation were reversibly depressed (up to 97%) by either adenosine (100 μM) or its non-hydrolysable analogue 2-chloroadenosine (CADO; 0·1–5·0 μM). These effects were often accompanied by postsynaptic hyperpolarization (up to 8 mV) and a reduction in input resistance (up to 11%). The selective adenosine A_1 receptor agonists 2-chloro-N^6-cyclopentyladenosine (CCPA; 0·1–0·4 μM) and $R(-)N^6$-(2-phenylisopropyl)-adenosine (R-PIA; 1 μM) both depressed the $EPSP_m$. In contrast, the adenosine A_{2A} receptor agonist 2-p-(2-carboxyethyl)-phenethylamino-5′-N-ethylcarboxamidoadenosine (CGS 21680; 0·5–1·0 μM) did not significantly affect the $EPSP_m$.

4. The selective adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0·2 μM) fully reversed the depressant effects of both adenosine (100 μM) and CADO (1 μM) on the $EPSP_m$ and the stimulus-evoked reductions in spike frequency adaptation.

5. DPCPX (0·2 μM) alone caused a small but variable mean increase in the $EPSP_m$ of 22 ± 19% and enabled activation of an $EPSP_m$ by a previously subthreshold stimulus. In contrast, the selective adenosine kinase inhibitor 5-iodotubercidin (5-IT; 10 μM) inhibited the $EPSP_m$ by 74 ± 10%, an effect that was reversed by DPCPX.

6. The concentration–response relationship for the depressant action of CADO on the $EPSP_m$ more closely paralleled that for its presynaptic depressant action on glutamate-mediated EPSPs than that for postsynaptic hyperpolarization. The respective mean IC_{50} and EC_{50} concentrations for these effects were 0·3, 0·8 and 3·0 μM.

7. CADO (1–5 μM) did not have a significant effect on the postsynaptic depolarization, increase in input resistance and reduction in spike frequency adaptation evoked by carbachol (0·5–3·0 μM). All these effects were abolished by atropine (1 μM).

8. These data provide good evidence for an adenosine A_1 receptor-mediated inhibition of mAChR-mediated synaptic responses in hippocampal CA1 pyramidal neurones. This inhibition is mediated predominantly presynaptically, is active tonically and can be enhanced when extracellular levels of endogenous adenosine are raised.

CA1 pyramidal neurones in the hippocampus receive multiple intrinsic synaptic inputs as well as a number of extrinsic synaptic inputs from diverse areas of the brain. One of the most characterized extrinsic inputs is the septohippocampal input (Dutar, Bassant, Senut & Lamour, 1995), which comprises a heterogeneous population of afferents that mediate their effects through the release of numerous neurotransmitters including γ-aminobutyric acid (GABA), acetylcholine (ACh) and a variety of neuropeptides (Decker & McGaugh, 1991; Dutar *et al.* 1995). Of these heterogeneous inputs the cholinergic aspect of the septohippocampal input to the hippocampus has probably received most attention due to its involvement in mnemonic processing (Cole & Nicoll, 1983; Decker & McGaugh, 1991; Dutar *et al.* 1995). This input has been shown to activate a slow excitatory postsynaptic potential (EPSP) and to reduce spike frequency adaptation (sometimes referred to as action potential accommodation) via muscarinic acetylcholine receptor (mAChR)-mediated inhibition of K^+ conductances (Cole & Nicoll, 1983, 1984; Madison, Lancaster & Nicoll, 1987; Segal, 1988; Pitler & Alger, 1990). This prolonged excitation of CA1 pyramidal neurones, if left unregulated, can be detrimental to these cells and may result in epileptogenesis (Lothman, Bertram & Stringer, 1991; Wasterlain, Fujikawa, Penix & Sankar, 1993). As such, mechanisms that control the magnitude of the mAChR-mediated postsynaptic responses are likely to be of major importance in maintaining the normal functioning of the CNS.

A common mechanism of control of synaptic inputs within the brain is via activation of different receptors from those that mediate the postsynaptic response, e.g. activation of autoreceptors, or presynaptic heteroreceptors (Thompson, Capogna & Scanziani, 1993). The aim of this study, therefore, was to investigate the possibility that a neurotransmitter/neuromodulator other than ACh may restrict the activation of the mAChR-mediated postsynaptic responses. We chose to study adenosine since this neuromodulator plays an important role in controlling the excitability of neuronal networks by inhibiting other excitatory synaptic inputs, e.g. glutamate (Thompson, Haas & Gähwiler, 1992; Thompson *et al.* 1993). To do this we isolated stimulus-evoked mAChR-mediated synaptic responses (i.e. the mAChR-mediated EPSP ($EPSP_m$) and the stimulus-evoked reduction in spike frequency adaptation in CA1 pyramidal neurones) using a cocktail of ionotropic glutamate and GABA receptor antagonists to block all 'fast' excitatory and inhibitory synaptic transmission. Using this approach we have been able to investigate how drugs that affect the function of adenosine at the levels of the receptor and its metabolism influence both the $EPSP_m$ and the mAChR-mediated reduction in spike frequency adaptation. Preliminary accounts of part of this work have appeared in abstract form (Morton & Davies, 1996*a*, *b*).

METHODS

Female Wistar rats (2–4 weeks old) were cervically dislocated and subsequently decapitated in accordance with UK Home Office guidelines. The brain was removed rapidly and transverse hippocampal slices were prepared by hemisecting the whole brain minus the cerebellum and cutting 400 μm thick transverse coronal slices containing hippocampal slices, using a vibroslicer (Campden Instruments, Loughborough, UK). The CA3 region of each slice was then cut away to eliminate changes in network function that can occur due to epileptiform bursting in area CA3 when picrotoxin is applied to the slice. The resultant CA3-ectomized slices were placed on a nylon mesh at the interface of a warmed (32–34 °C), perfusing (1–2 ml min^{-1}) artificial cerebrospinal fluid and an oxygen-enriched (95% O_2–5% CO_2) humidified atmosphere. The standard perfusion medium comprised (mM): NaCl, 124; KCl, 3; $NaHCO_3$, 26; NaH_2PO_4, 1·25; $CaCl_2$, 2; $MgSO_4$, 1; D-glucose, 10; and was bubbled with 95% O_2–5% CO_2.

Following a 1 h equilibration period, intracellular recordings were obtained from the CA1 pyramidal cell body region using 2 M potassium methylsulphate-filled microelectrodes (60–110 MΩ). An Axoclamp-2B amplifier (Axon Instruments) was used in discontinuous (3–5 kHz switching frequency) current clamp mode. All impalements were made in control medium. Once stable recordings had been made for at least 10 min, all fast ionotropic glutamate receptor-mediated synaptic transmission was blocked using a combination of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μM; Tocris Cookson Ltd, Bristol, UK) or 6-nitro-7-sulphamoylbenzo(*f*)quinoxaline-2,3-dione (NBQX; 2–4 μM; Tocris Cookson Ltd), and the *N*-methyl-D-aspartate (NMDA) receptor antagonists D-(*E*)-2-amino-4-methyl-5-phosphono-3-pentanoic acid (CGP 40116; 50 μM; Ciba-Geigy Ltd) or D-2-amino-5-phosphonopentanoate (AP5; 50 μM; Tocris Cookson Ltd), and all GABA-mediated synaptic transmission was abolished using the $GABA_A$ receptor antagonist picrotoxin (50 μM; Sigma) and the $GABA_B$ receptor antagonist 3-*N*-[1-(*S*)-(3,4-dichlorophenyl)ethyl]amino-2-(*S*)-hydroxypropyl-*p*-benzyl-phosphonic acid (CGP 55845A; 1 μM; Ciba-Geigy Ltd). Bipolar stimulating electrodes, made from 55 μm diameter insulated nickel–chromium wire, were positioned in the stratum oriens close to the recording electrode in the stratum pyramidale (Fig. 1*A*) to provide extracellular orthodromic activation of CA1 neurones. In each series of experiments, stimuli comprised square-wave pulses (20–200 μs; 5–30 V) delivered homosynaptically at a fixed intensity every 5–10 min. Data were captured using pCLAMP 6 software (Axon Instruments) and digitized records were stored on the hard disk of a PC and on DAT tape (DTR-1404; Biologic Scientific Instruments, Claix, France) for off-line analysis using Clampfit software (Axon Instruments). During the period between stimuli the input resistance and the extent of spike frequency adaptation of each neurone were measured routinely every 2 min using 300–600 ms long hyperpolarizing and depolarizing current steps (±0·15–0·40 nA), respectively. In all experiments in which $EPSP_m$s or stimulation-induced reductions in spike frequency adaptation were evoked, baseline recordings comprised either three successive $EPSP_m$s which had peak amplitudes that differed by no more than 15%, or reductions in spike frequency adaptation that were consistent over 20–30 min, respectively. All drugs were applied by addition to the perfusion medium. To compare the $EPSP_m$s evoked in the presence and absence of a drug at the same membrane potential, DC was injected through the

electrode to compensate for any drug-induced changes in membrane potential. 2-*p*-(2-Carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS 21680), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 5-iodotubercidin (5-IT), (+)-α-methyl-4-carboxyphenylglycine ((+)-MCPG) and nitrendipine were obtained from Research Biochemicals International. Adenosine, atropine, carbachol, 2-chloroadenosine (CADO), 2-chloro-N^6-cyclopentyladenosine (CCPA), $R(-)N^6$-(2-phenylisopropyl)-adenosine (R-PIA), physostigmine and picrotoxin were purchased from Sigma.

Data are presented as means ± standard error of the mean (S.E.M.) and statistical significance was assessed using Student's paired or unpaired *t* tests performed on raw data with $P < 0.05$ being taken as indicating statistical significance. *n* values refer to the number of times a particular experiment was performed, each in a different slice taken from a different rat.

RESULTS

Data were obtained from 168 stable intracellular recordings (2–6 h) from CA1 pyramidal neurones with overshooting action potentials, resting membrane potentials that were more negative than −55 mV and input resistance values of 30 MΩ or greater.

Characterization of a slow EPSP

Single shock stimulation in the stratum oriens evoked an EPSP which was followed by a biphasic inhibitory postsynaptic potential (IPSP). All components of this response were abolished by the combined application of NBQX (1–3 μM) or CNQX (20 μM), AP5 (50 μM) or CGP 40116 (50 μM), picrotoxin (50 μM) and CGP 55845A (1 μM) (Fig. 1*B*). A 2- to 10-fold increase in the stimulus intensity evoked a much slower EPSP in 126 out of 129 neurones in which this was attempted (Fig. 1*C*). This EPSP could be evoked reproducibly every 5–10 min. In a random group of cells the slow EPSP had a mean amplitude of 9·6 ± 2·1 mV, a time to peak of 2·6 ± 0·4 s and an overall duration of 32·6 ± 6·9 s ($n = 6$). The peak amplitude and duration of the EPSP were highly dependent upon the stimulus strength used, both increasing with increasing stimulus intensity (Fig. 1*C*). The EPSP often resulted in intense discharges of action potentials and in some neurones the EPSP depolarized the membrane beyond the action potential firing threshold and into a region where action potential generation was inactivated. The slow EPSP was accompanied by an increase in cell input resistance as measured by comparing responses to hyperpolarizing current steps applied to the cell before and during the EPSP (Fig. 1*D*).

As illustrated in Fig. 2*A*, the slow EPSP was not affected significantly by the metabotropic glutamate receptor antagonist (+)-MCPG (1000 μM; $n = 3$). Addition of the acetylcholinesterase inhibitor physostigmine (1–5 μM), however, caused a large enhancement of both the peak amplitude and duration of the slow EPSP ($n = 5$; Fig. 2*B*). Physostigmine also caused a reduction in spike frequency adaptation as measured by the number of action potentials fired in response to a 300 ms long +0·2 or +0·3 nA current step ($n = 6$; not illustrated). This effect presumably occurred as a result of raising extracellular levels of spontaneously released ACh due to inhibition of acetylcholinesterase activity by physostigmine (Cole & Nicoll, 1984; Azouz, Jensen & Yaari, 1994). In contrast, atropine (2–5 μM) reduced or abolished the slow EPSP ($n = 7$; Fig. 2*C*). These results are entirely consistent with the slow EPSP being mediated by activation of mAChRs and, as such, this EPSP will be referred to as a mAChR-mediated EPSP ($EPSP_m$).

Effects of adenosine receptor activation on the $EPSP_m$

We next addressed whether activation of adenosine receptors might affect the $EPSP_m$. The non-hydrolysable adenosine receptor agonist CADO (0·2–1 μM) caused a depression of the $EPSP_m$ that was maintained for the period of the agonist application and was fully reversed on washout ($n = 4$; Fig. 3*A*). This effect was concentration dependent and, at a concentration of 0·2 μM, CADO sometimes caused a substantial depression of the $EPSP_m$ without any effect on the postsynaptic membrane potential, input resistance (Fig. 3*B*) or spike frequency adaptation in response to a depolarizing current step. In contrast, at concentrations of 0·5 μM and above the depressant action of CADO on the $EPSP_m$ was invariably accompanied by postsynaptic hyperpolarization (up to 8 mV) and a reduction in input resistance (up to 11%). In one neurone the $EPSP_m$ was unaffected by CADO. The maximal effect of CADO on the $EPSP_m$ was 97% inhibition. Similar results were obtained using adenosine itself. Thus, at a concentration of 100 μM, adenosine inhibited the $EPSP_m$ by 78 ± 10% ($n = 3$).

Pharmacology of the adenosine receptor mediating the depression of the $EPSP_m$

To elucidate which adenosine receptor subtype mediated the CADO-induced depression of the $EPSP_m$, we investigated the effects of a number of adenosine receptor subtype-specific agonists. The adenosine A_1 receptor agonists CCPA (0·1–0·4 μM; $n = 7$; Fig. 4*A*) and R-PIA (1 μM; $n = 2$) depressed the $EPSP_m$ and caused postsynaptic hyperpolarizations associated with reductions in input resistance. Thus, at a concentration of 0·4 μM, CCPA depressed the peak amplitude of the $EPSP_m$ to 34 ± 6% of that in control medium and produced an accompanying hyperpolarization of 4–5 mV and a reduction in input resistance of 5–10% ($n = 4$). In contrast, the adenosine A_{2A} receptor agonist CGS 21680, at concentrations up to 1 μM, neither affected the $EPSP_m$ nor the passive membrane properties of the neurones in which it was tested ($n = 3$; Fig. 4*B*).

The effects of the selective adenosine A_1 receptor antagonist DPCPX (0·2 μM) were also tested. DPCPX fully reversed, or prevented, the inhibition of the $EPSP_m$ (Fig. 5*A*) as well as the postsynaptic hyperpolarizations and decreases in input resistance (see Fig. 8*A*) evoked by CADO (1–2 μM; $n = 8$) and adenosine (100 μM; $n = 3$; not illustrated).

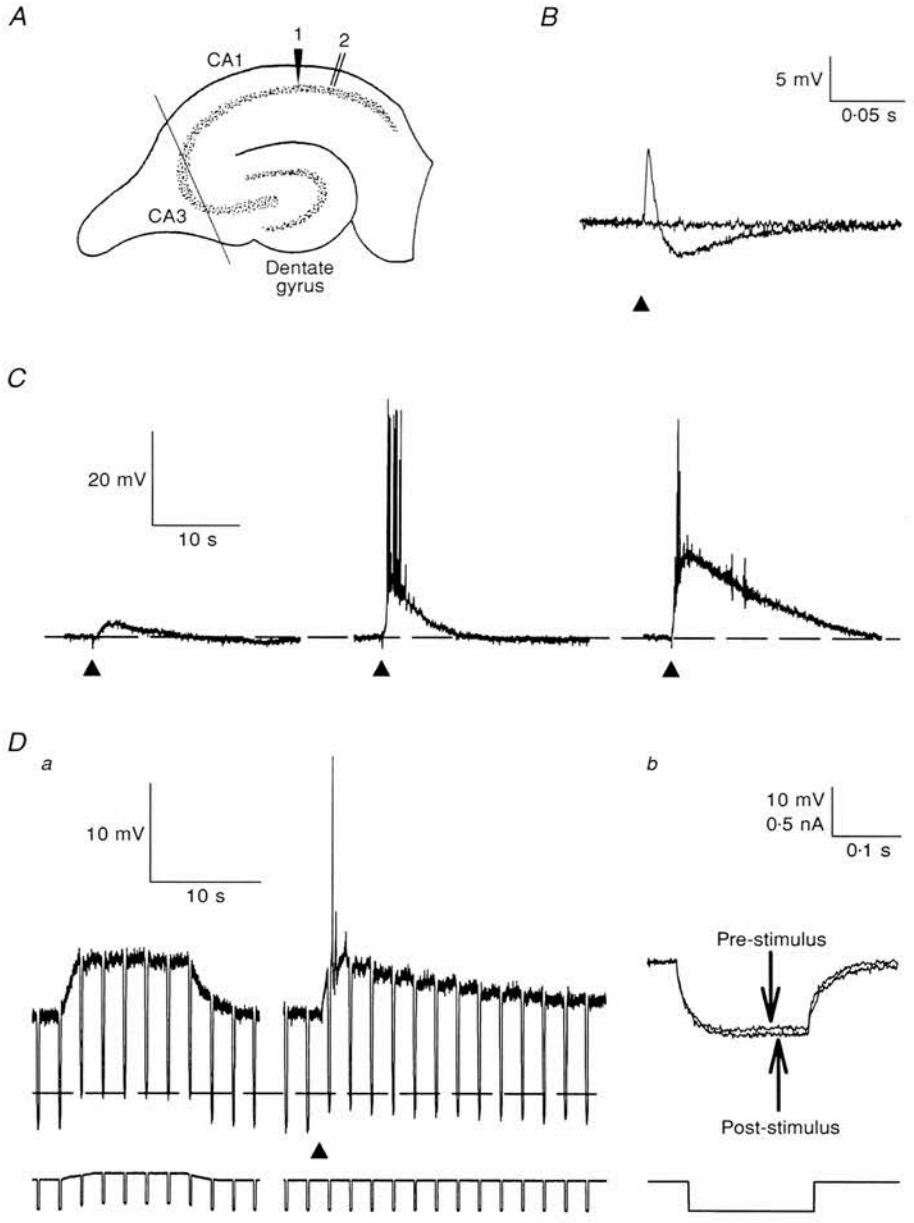

Figure 1. Characteristics of the pharmacologically isolated slow EPSP evoked in response to a single high intensity stimulus in the stratum oriens

A, schematic diagram of a rat hippocampal slice illustrating the positioning of recording (1) and stimulating (2) electrodes required to evoke the synaptic responses in *B–D*. *B*, intracellular recordings from a CA1 neurone in response to single shock stimulation in the stratum oriens. Note that in control medium an EPSP is followed by an IPSP and that the combination of CNQX (20 μM), CGP 40116 (50 μM), picrotoxin (50 μM) and CGP 55845A (1 μM) abolished this response (superimposed horizontal line). Traces are averages of four successive records taken at the same membrane potential. In *C*, the traces are single sweeps which illustrate the stimulus-dependent activation of a slow EPSP in the presence of the four amino acid receptor antagonists listed above. The stimulus intensities used to evoke the responses from left to right were 1·8, 2·3 and 4 mA, respectively. (The intensity that was required to evoke an AMPA/kainate receptor-mediated EPSP that was just suprathreshold for evoking an action potential was 0·04 mA.) The action potentials evoked by the slow EPSP are truncated due to the low sampling rate used to capture the entire response. The membrane potential of this neurone was −64 mV. *D*, the increase in input resistance associated with the slow EPSP. The input resistance was measured from the change in membrane potential in response to hyperpolarizing current steps of −0·3 nA applied for 0·2 s every 2·0 s. In *Da*, the cell membrane potential was manually clamped at a depolarized level before the stimulus so that the voltage response to the hyperpolarizing current steps could be compared with that evoked near the peak of the slow EPSP. *Db*, comparison of the responses in *a* on an expanded time scale. The current injected into the cell is shown below the voltage traces. The initial membrane potential of this cell was −64 mV. In this and all subsequent figures, the arrowheads below each synaptic trace indicate the time of afferent stimulation.

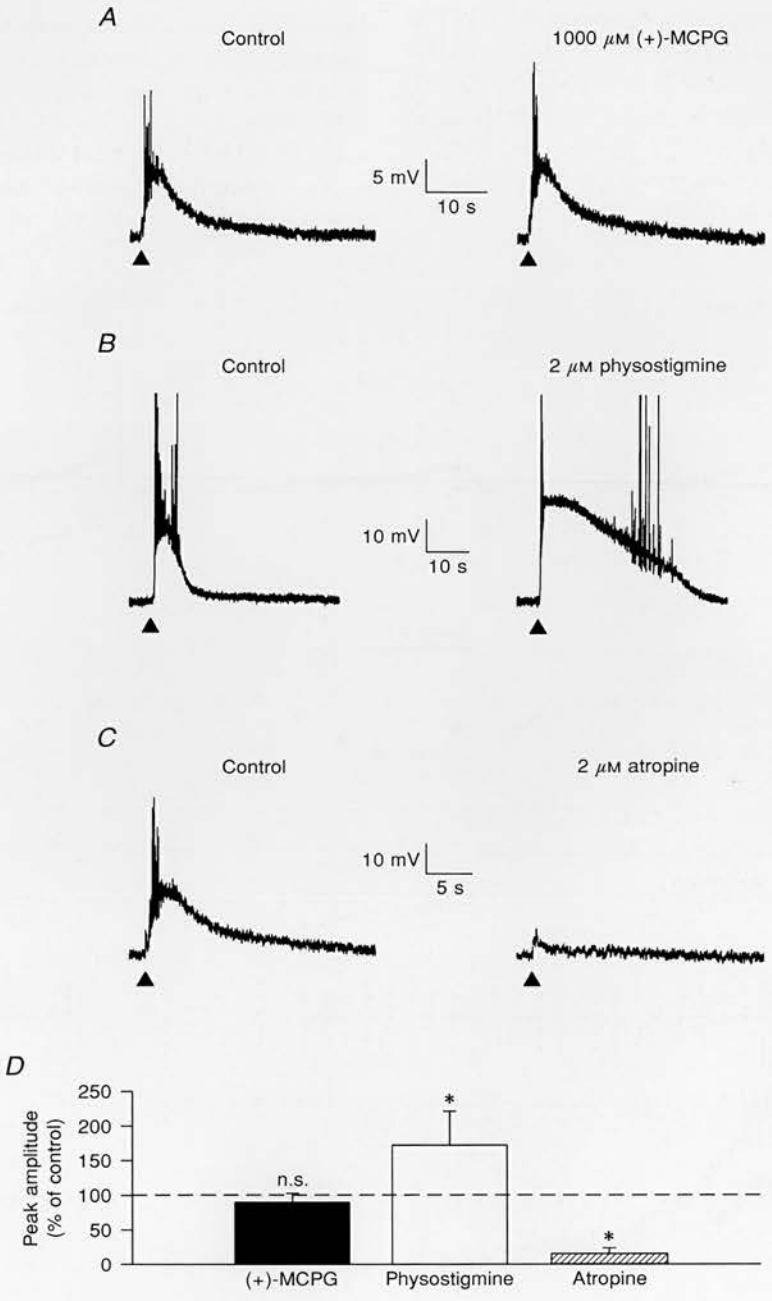

Figure 2. Effects of (+)-MCPG, physostigmine and atropine on the slow EPSP

In *A–C*, each trace is a single sweep illustrating the effects of 1000 μM (+)-MCPG, 2 μM physostigmine and 2 μM atropine, respectively, on slow EPSPs evoked in three separate neurones. The membrane potentials of these cells were −63, −64 and −63 mV, respectively. *D*, bar graph in which the pooled data for the peak amplitude of $EPSP_m$s recorded after 15–30 min in the presence of either (+)-MCPG (1000 μM; $n = 3$), physostigmine (2 μM; $n = 3$) or atropine (2 μM; $n = 3$) is expressed as a percentage of the mean value of the control $EPSP_m$s recorded over a 20–30 min baseline period prior to each drug application. Values are means ± S.E.M.; 100% is equivalent to no change. Note that whilst (+)-MCPG did not have a significant effect on the slow EPSP, physostigmine significantly enhanced the slow EPSP and atropine significantly inhibited the slow EPSP. $*P < 0{\cdot}05$; n.s., not significant (compared with control). In this and all subsequent figures, unless stated otherwise, traces are individual synaptic responses recorded intracellularly in response to a single stimulus delivered in the stratum oriens in the presence of 2–4 μM NBQX, 50 μM CGP 40116, 50 μM picrotoxin and 1 μM CGP 55845A. Each sweep was taken at the same membrane potential, which was achieved using DC injection to compensate for any drug-induced changes.

DPCPX alone caused a small but variable increase in the size of the $EPSP_m$ in three out of four neurones studied: the peak amplitude of the $EPSP_m$ in the presence of DPCPX was $122 \pm 19\%$ that of control ($n = 4$). In addition, in the presence of DPCPX, a stimulus that was previously sufficient to reduce spike frequency adaptation but was either subthreshold or just suprathreshold for evoking an $EPSP_m$ subsequently evoked a greatly enhanced $EPSP_m$ that had a peak amplitude of 8–10 mV ($n = 2$; Fig. 5*B*).

Effects of CADO on the reduction in spike frequency adaptation evoked by endogenous ACh

Stimulation that was subthreshold for activating the $EPSP_m$ caused a reduction in spike frequency adaptation in response to a depolarizing current step delivered 2 s after pathway stimulation ($n = 12$; Figs 5*B* and 6*A*). This reduction in spike frequency adaptation was inhibited by CADO (Fig. 6*B*) in a concentration-dependent manner (1–5 μM; $n = 3$) and was reinstated by subsequent application of DPCPX (0·2 μM;

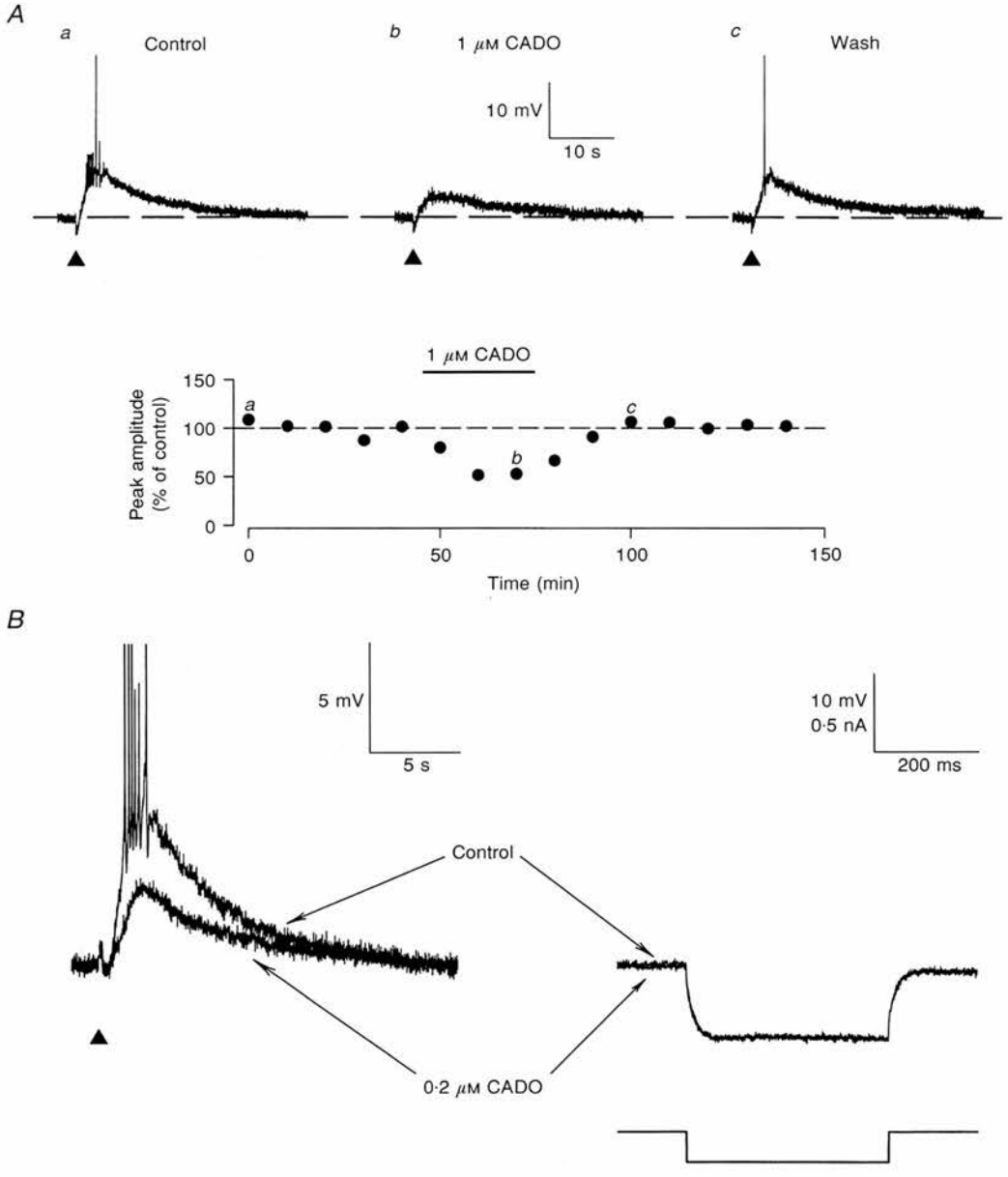

Figure 3. The effect of CADO on the $EPSP_m$

In *A*, synaptic traces are $EPSP_m$s, evoked in response to a single stimulus, recorded in control medium (*a*), in the presence of 1 μM CADO (*b*) and following washout (*c*). The graph shows a plot of the peak amplitudes of successive $EPSP_m$s normalized to the mean peak amplitude of the five $EPSP_m$s prior to CADO application *versus* time for a single experiment. CADO was applied for the time indicated by the bar. *a*, *b* and *c* refer to the synaptic traces illustrated above the graph. *B*, superimposed traces of $EPSP_m$s (left) and responses to a −0·2 nA current step (right) in the presence and absence of 0·2 μM CADO. Note that 0·2 μM CADO caused a substantial reduction in the $EPSP_m$ without affecting the input resistance of the cell. The membrane potential of both cells was −64 mV.

$n = 3$; Fig. 6*C*). In two neurones, in the combined presence of CADO and DPCPX, a small $EPSP_m$ was evoked by the previously subthreshold stimulus. This $EPSP_m$, along with the reduction in spike frequency adaptation, was abolished by subsequent application of atropine (1 μM; Fig. 6*D*).

Effects of an adenosine kinase inhibitor on the $EPSP_m$

The increase in the $EPSP_m$ that was caused by DPCPX alone suggested that endogenous adenosine within the slice was capable of tonically activating the adenosine A_1 receptors which inhibit the $EPSP_m$. Therefore, we next examined whether it was possible to potentiate this tonic effect by impairing the breakdown of adenosine. To do this we investigated the effects of the selective adenosine kinase inhibitor 5-IT which raises extracellular adenosine levels by preventing the breakdown of adenosine into adenosine monophosphate (AMP) (Pak, Haas, Decking & Schrader, 1994). 5-IT (10 μM) caused a postsynaptic hyperpolarization of 2–8 mV, a decrease in input resistance of 5–15% and a 74 ± 10% depression of the $EPSP_m$ ($n = 4$; Fig. 7*B*). In

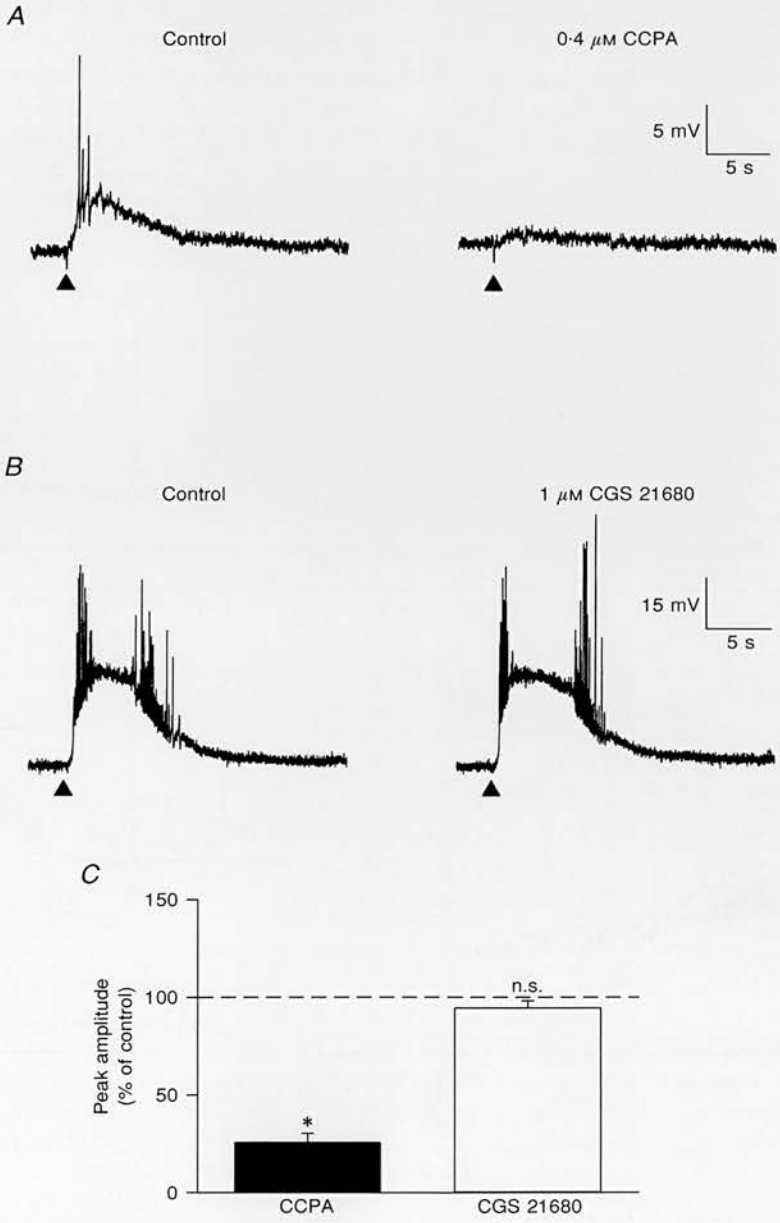

Figure 4. Effects of subtype-selective adenosine receptor agonists on the $EPSP_m$

In *A* and *B*, traces illustrate the respective effects of 0·4 μM CCPA and 1 μM CGS 21680 on $EPSP_m$s evoked in separate neurones. The membrane potentials of these cells were −63 and −64 mV, respectively. *C*, bar graph in which pooled data for the effects of CCPA (0·4 μM; $n = 4$) and CGS 21680 (0·5 μM; $n = 3$) on the $EPSP_m$ are plotted as described in Fig. 2*D*. Note that whilst CGS 21680 did not have a significant effect, CCPA significantly inhibited the $EPSP_m$. Means ± S.E.M.; * $P < 0{\cdot}05$; n.s., not significant (compared with control).

three of these neurones, subsequent application of DPCPX (0·2 μM) completely reversed these effects (Fig. 7*A*).

Is the adenosine-mediated depression of mAChR-mediated postsynaptic responses pre- or postsynaptic?

Having established a depressant action of adenosine on postsynaptic mAChR-mediated responses evoked by afferent stimulation, we addressed next whether these effects were mediated pre- or postsynaptically. Initially, we compared the concentration–response relationships for the depression of the $EPSP_m$ with those of known post- and presynaptic adenosine A_1 receptor-mediated effects, i.e. membrane hyperpolarization (Fig. 8*A*; Greene & Haas, 1985; Gerber, Greene, Haas & Stevens, 1989) and depression of glutamate AMPA/kainate receptor-mediated EPSPs (Fig. 8*B*; Lupica, Proctor & Dunwiddie, 1992), respectively. As illustrated in Fig. 8*C*, CADO was more potent at inhibiting the $EPSP_m$ than it was at inhibiting pure AMPA/kainate receptor-mediated EPSPs or causing postsynaptic hyperpolarization. The respective IC_{50} values and EC_{50} value for these effects were 0·3, 0·8 and 3·0 μM (Fig. 8*C*). In addition, the logistic fit for the depressant action of CADO on the $EPSP_m$ more closely paralleled that for its presynaptic depressant action on glutamate-mediated EPSPs.

If CADO was acting presynaptically to depress both the $EPSP_m$ and the reduction in spike frequency adaptation evoked by subthreshold stimulation then it should not affect the respective postsynaptic responses evoked by carbachol. To maximize the probability of observing an effect of CADO on carbachol-induced postsynaptic responses, we used concentrations of carbachol that (i) were close to the EC_{50} values reported for causing postsynaptic depolarization and

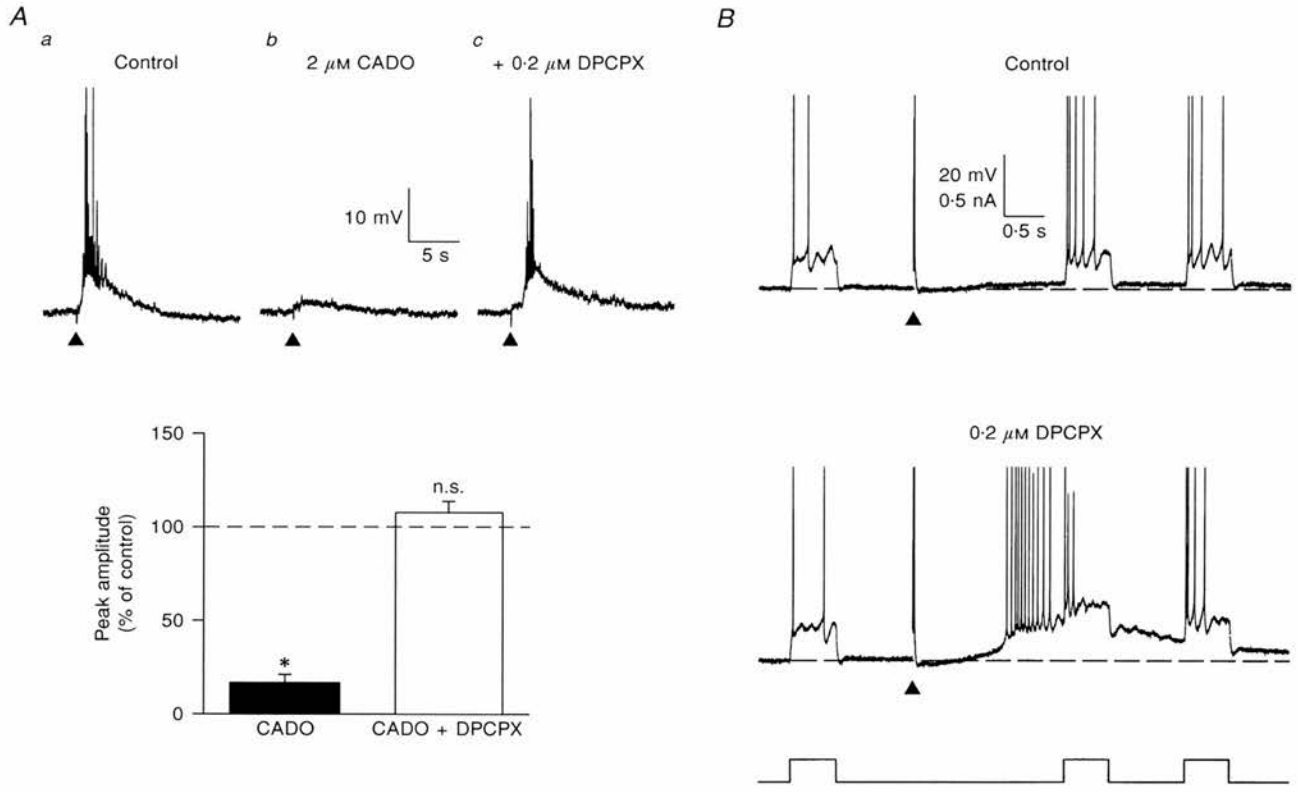

Figure 5. Effects of DPCPX on the $EPSP_m$ and the CADO-induced depression of the $EPSP_m$

In *A*, synaptic traces are $EPSP_m$s recorded in control medium (*a*), in the presence of 2 μM CADO (*b*) and in the combined presence of 2 μM CADO and 0·2 μM DPCPX (*c*). The membrane potential of the cell was −64 mV. The bar graph illustrates pooled data for the effects of 1 μM CADO ($n = 8$) and 1 μM CADO + 0·2 μM DPCPX ($n = 4$) on the $EPSP_m$. Each value was calculated as described for Fig. 2*D*. Note that CADO significantly depressed the $EPSP_m$ and that responses fully recovered in the additional presence of both CADO and DPCPX. Means ± S.E.M.; $*P < 0·05$; n.s., not significant. In *B*, traces are continuous records of the membrane potential of a single cell in which depolarizing current steps (+0·2 nA, 600 ms) were delivered 1 s prior to, and 2 and 3·5 s after pathway stimulation at an intensity just suprathreshold for activating the $EPSP_m$ in medium containing the four glutamate and GABA receptor antagonists (top trace) and following the addition of 0·2 μM DPCPX (middle trace). The bottom trace represents the current injected into the cell. The membrane potential of the cell was −65 mV. Note that in control medium spike frequency adaptation was reduced following pathway stimulation. After addition of DPCPX this intensity of stimulation now additionally activated a substantial $EPSP_m$.

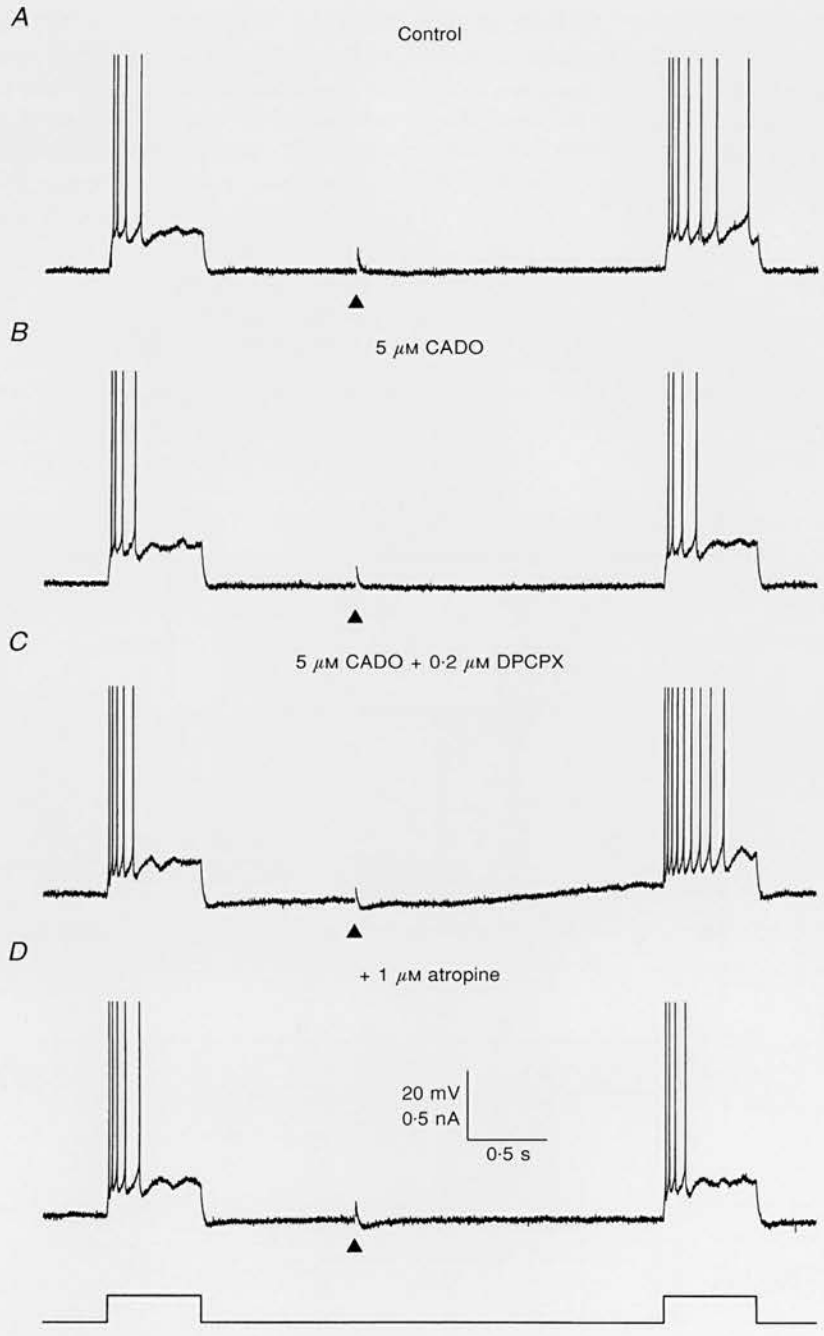

Figure 6. CADO depressed the reduction in spike frequency adaptation evoked by pathway stimulation that was subthreshold for activating the $EPSP_m$

Traces were generated as described in Fig. 5*B* except that only the response to the first depolarizing current step after afferent stimulation is illustrated. The responses were obtained in medium containing the four glutamate and GABA receptor antagonists (*A*), in the additional presence of 5 μM CADO (*B*), in the combined presence of 5 μM CADO and 0·2 μM DPCPX (*C*) and following subsequent addition of 1 μM atropine (*D*). The point of pathway stimulation is indicated by the arrowheads and the trace at the bottom of the figure illustrates the points at which current was injected into the cell. Note that in control medium spike frequency adaptation was reduced following pathway stimulation, that this reduction was abolished by CADO in a DPCPX-sensitive manner and that it was also completely inhibited by subsequent addition of atropine. If two current steps were delivered without intervening subthreshold afferent stimulation, the level of spike frequency adaptation evoked by the second step was not significantly different from that evoked by the first step ($n = 4$; $P > 0{\cdot}05$; not illustrated). Note that in the presence of CADO + DPCPX previously subthreshold pathway stimulation now evoked a small $EPSP_m$ that was abolished by the subsequent addition of atropine. The initial membrane potential of the neurone was −66 mV.

reduction in spike frequency adaptation (Madison *et al.* 1987), and (ii) produced responses similar to those evoked by afferent stimulation (see Fig. 10). In addition, we used concentrations of CADO that were near-maximal for inhibiting the $EPSP_m$ and the reduction in spike frequency adaptation evoked by subthreshold stimulation (see Figs 6 and 8*C*).

In a first series of experiments, we tested the effect of CADO on the postsynaptic depolarization and increase in input resistance evoked by brief bath applications of carbachol (3 μM for 30–60 s). In four neurones, repeated application of carbachol caused consistent and reversible depolarizations that were associated with increases in input resistance (Figs 9*A* and 10*A*). In these same neurones, addition of CADO (1 μM) caused a hyperpolarization (1–5 mV) that was associated with a small decrease in input resistance (4–7%). However, CADO did not have a significant effect on the carbachol-induced depolarizations and increases in input resistance ($n = 4$; $P > 0{\cdot}05$; Fig. 9*A*). In five other neurones, these postsynaptic effects of carbachol were abolished by atropine (1–5 μM; not illustrated). These results contrast with those for the $EPSP_m$, which was depressed significantly by both CADO (1 μM, $P < 0{\cdot}05$; Fig. 10*A*) and atropine (1–5 μM, $P < 0{\cdot}05$; Fig. 2*C*).

In a second series of experiments, we investigated the effects of CADO and carbachol on spike frequency adaptation

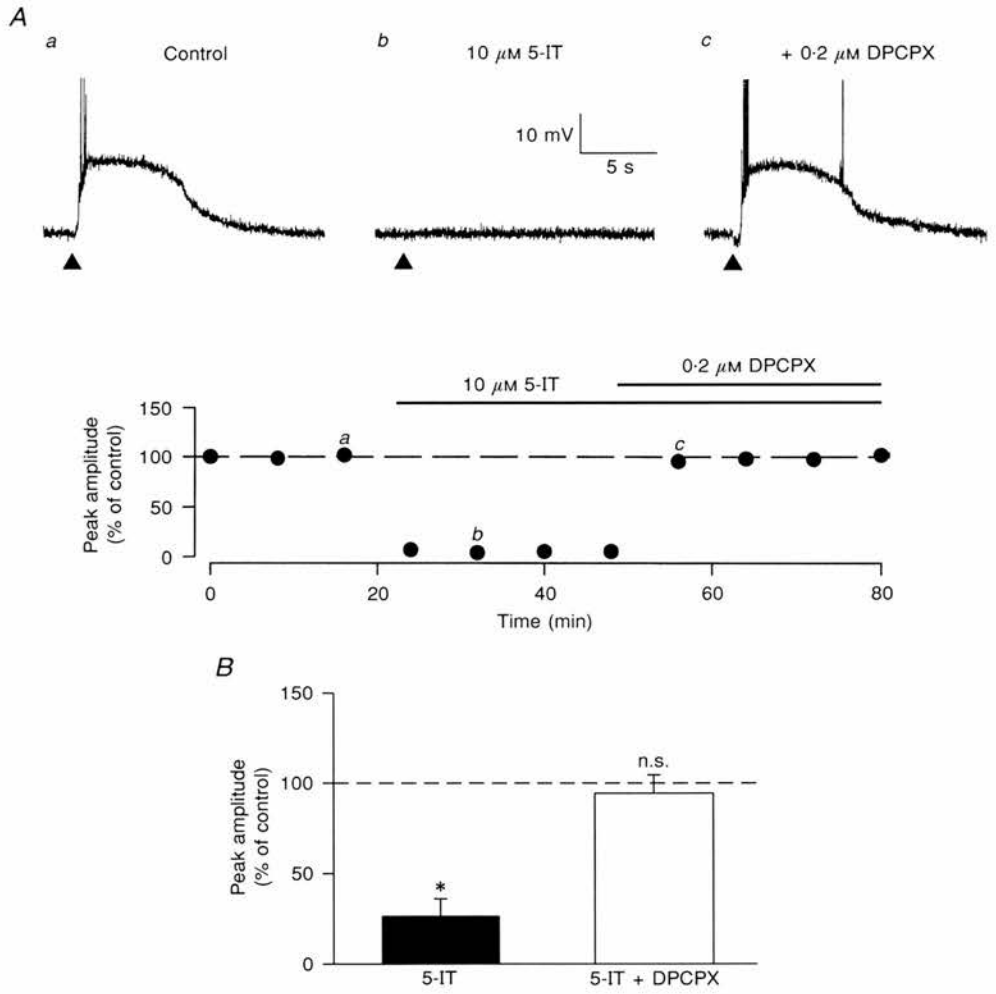

Figure 7. The effects of an inhibitor of adenosine kinase on the $EPSP_m$

A, the bottom graph is similar to that illustrated in Fig. 3 and shows the peak amplitude of successive $EPSP_m$s, for a single experiment, *versus* time to illustrate the depressant effect of 10 μM 5-IT on the $EPSP_m$ and its reversal by 0·2 μM DPCPX. The duration of application of each drug is indicated by the bars above the graph. The synaptic traces (top) are representative $EPSP_m$s recorded in control (*a*), in the presence of 5-IT (*b*) and in the presence of 5-IT + DPCPX (*c*) taken at the time points indicated. The membrane potential of this neurone was −66 mV. *B*, bar graph illustrating pooled data for the effects of 5-IT (10 μM; $n = 4$), and 5-IT + 0·2 μM DPCPX ($n = 3$) on the $EPSP_m$. The values plotted were calculated as described in Fig. 2*D*. Note that 5-IT significantly depressed the $EPSP_m$ and that responses in the presence of both 5-IT and DPCPX were not significantly different from controls. Means ± S.E.M.; $*P < 0{\cdot}05$; n.s., not significant.

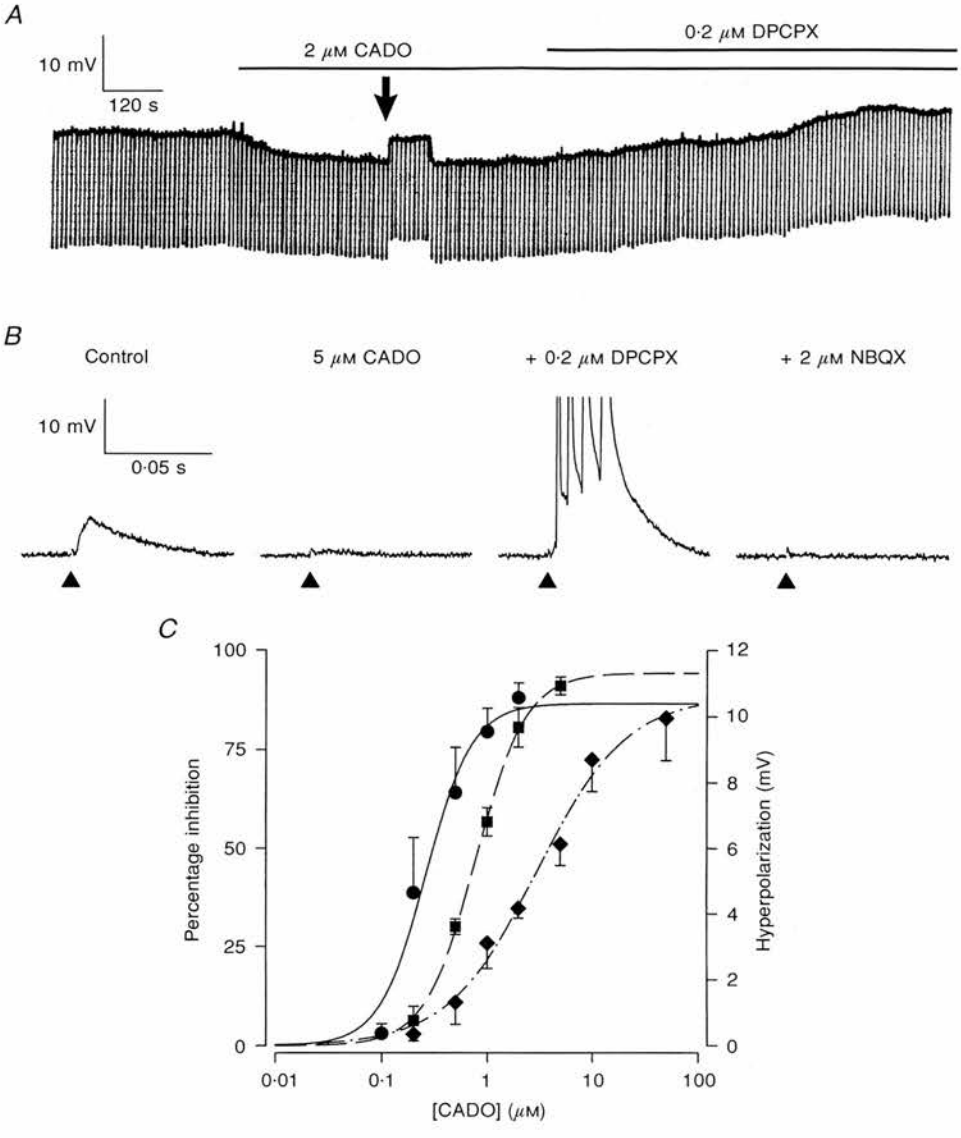

Figure 8. Comparison of the concentration–response relationships for CADO-induced depression of the $EPSP_m$ with those for postsynaptic hyperpolarization and depression of glutamate-mediated EPSPs

A, continuous chart record illustrating the effects of 2 μM CADO and 0·2 μM DPCPX on passive membrane properties of a CA1 neurone. The trace shows the membrane potential (thick line) and hyperpolarizing voltage responses (downward deflections) of the cell to constant current steps (−0·3 nA, 300 ms). The arrow indicates the point at which DC was injected for 90 s to reset the membrane potential of the cell to the level prior to CADO application. Note: (i) that CADO caused a hyperpolarization and a reduction in input resistance that was completely reversed by DPCPX, and (ii) that in the presence of DPCPX the membrane potential became more depolarized than that before any drug treatment, presumably indicating the presence of an endogenous adenosine tonus in the slice. The membrane potential of this neurone was −62 mV. In *B*, the responses are pure AMPA/kainate receptor-mediated EPSPs ($EPSP_A$s) recorded in the combined presence of 50 μM CGP 40116, 50 μM picrotoxin and 1 μM CGP 55845A. The traces, from left to right, are representative $EPSP_A$s recorded in control medium, in the presence of 5 μM CADO, in the combined presence of 5 μM CADO and 0·2 μM DPCPX and following subsequent addition of 2 μM NBQX. Note that DPCPX reversed the CADO-induced depression of the $EPSP_A$ and that in the presence of DPCPX the $EPSP_A$ gave rise to multiple action potentials. This increase in magnitude above control levels again presumably reflects the presence of an endogenous adenosine tonus in the slice. Each trace is an average of four successive responses evoked 15 s apart and the membrane potential of this neurone was −70 mV. *C*, plots of the percentage inhibition of $EPSP_m$s (●; data from 19 neurones) and of $EPSP_A$s (■; data from 4 cells) induced by CADO *versus* concentration of CADO. Superimposed on these plots is a plot of the magnitude of CADO-induced postsynaptic hyperpolarization, from a starting membrane potential of between −62 and −64 mV, *versus* concentration of CADO (◆; data from 18 neurones). Each point for each of the three plots is the mean value obtained from three to nine separate neurones and the error bars represent S.E.M. All data (Y) were fitted to the logistic expression: $Y = M(X^P/(X^P + K^P))$, where X is the concentration of CADO, M is the maximum effect, K is the IC_{50} or EC_{50} value and the power P determines the slope of the sigmoidal curve.

evoked in response to a depolarizing current step. Carbachol (0·5 μM) caused a small depolarization and a reduction in spike frequency adaptation that was reversible on washout ($n = 5$). Subsequent addition of CADO (5 μM) slightly, but not significantly, enhanced the level of spike frequency adaptation *per se* but did not significantly affect the reduction in spike frequency adaptation induced by a second application of carbachol ($n = 3$; Fig. 9*B*). In contrast, atropine abolished the carbachol-induced reduction in spike frequency adaptation ($n = 5$; not illustrated). These results differ from those in which spike frequency adaptation was reduced using subthreshold stimulation in that the latter was abolished by both CADO (5 μM; Fig. 10*B*) and atropine (1 μM).

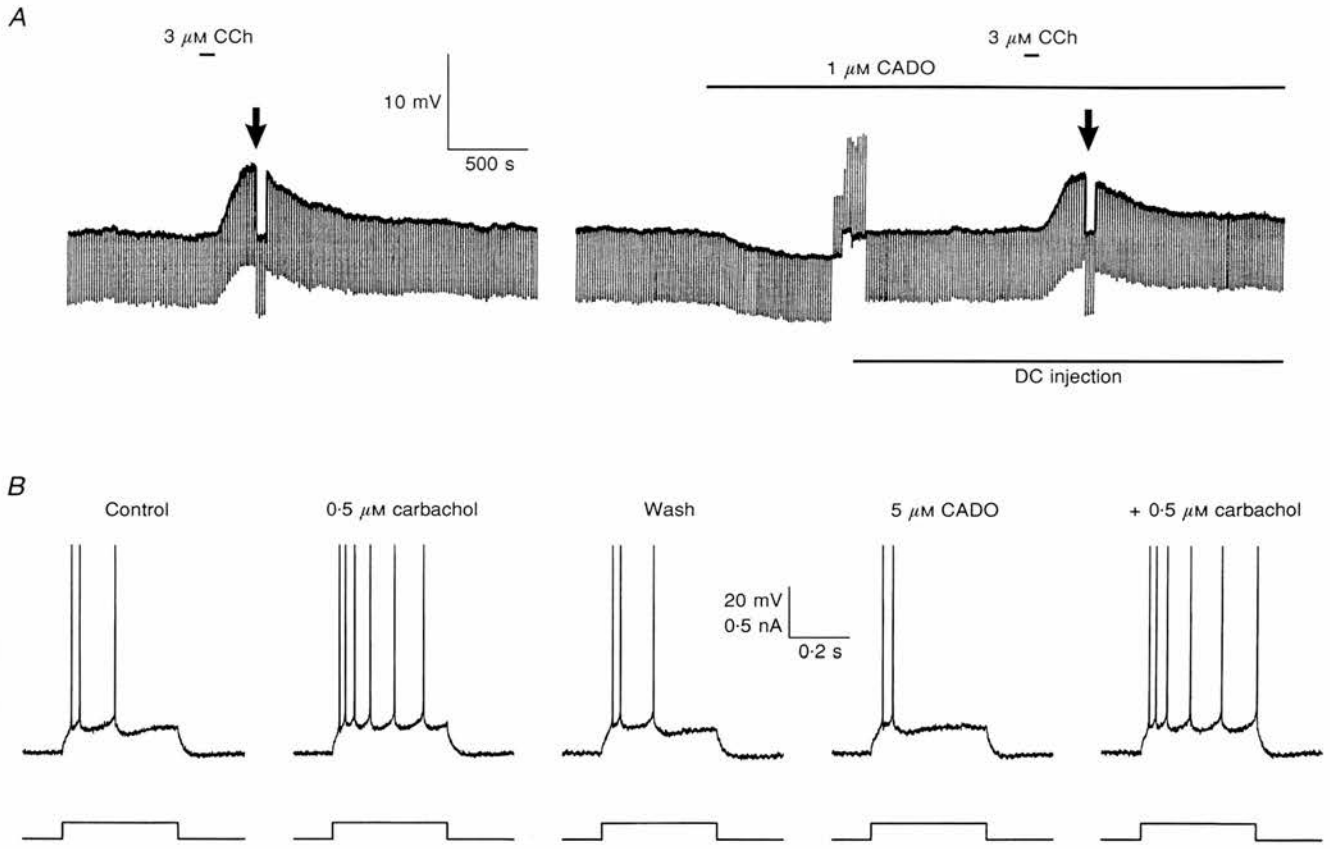

Figure 9. The effects of CADO on postsynaptic responses evoked by carbachol

A shows the effect of CADO (1 μM) on the change in membrane potential and input resistance evoked by carbachol (CCh; 3 μM). The trace is a chart record of membrane potential and cell input resistance, as described in Fig. 8, and illustrates the effects of carbachol on these parameters prior to and following CADO application. The arrows just after the end of each carbachol application indicate the points at which DC was injected for 60 s to reset the membrane potential of the cell to the level prior to the application of the agonist. The bar below the chart record indicates the period during which DC injection was used in the presence of CADO to restore the membrane potential of the cell to that prior to CADO application. This was kept constant for the period indicated by the length of the bar except for when the increase in input resistance evoked by carbachol was measured. The upward deflections just prior to and just after the point at which DC was applied in the presence of CADO represent voltage responses to depolarizing current steps (+0·3 nA, 300 ms). The bars above the chart record represent the periods during which carbachol and CADO were applied. The initial membrane potential of this neurone was −64 mV. *B*, responses (from left to right) evoked by a depolarizing current step (+0·2 nA, 400 ms) in control medium, in the presence of carbachol (0·5 μM), following washout, in the presence of CADO (5 μM) and in the combined presence of CADO (5 μM) and carbachol (0·5 μM). The membrane potential of the cell was maintained at −66 mV throughout the experiment by injecting DC through the recording electrode to compensate for the hyperpolarizing and depolarizing effects of CADO and carbachol, respectively. The trace at the bottom of the figure represents the time during which depolarizing current steps were injected via the recording electrode. Note that carbachol reduced spike frequency adaptation evoked during the depolarizing step and that this was unaffected by CADO.

DISCUSSION

The mAChR-mediated EPSP

mAChR-mediated EPSPs have been recorded previously from CA1 pyramidal neurones most commonly in response to high frequency bursts of stimuli, e.g. 20–100 Hz for 0·5–1·0 s (Cole & Nicoll, 1984; Madison *et al.* 1987; Segal, 1988; Pitler & Alger, 1990; Azouz *et al.* 1994). These EPSPs were often evoked in the presence of acetylcholinesterase inhibitors which prolong the effective synaptic concentration of ACh. In addition, ionotropic glutamate and GABA receptor antagonists were not added to the perfusion medium and, therefore, mAChR-mediated EPSPs were

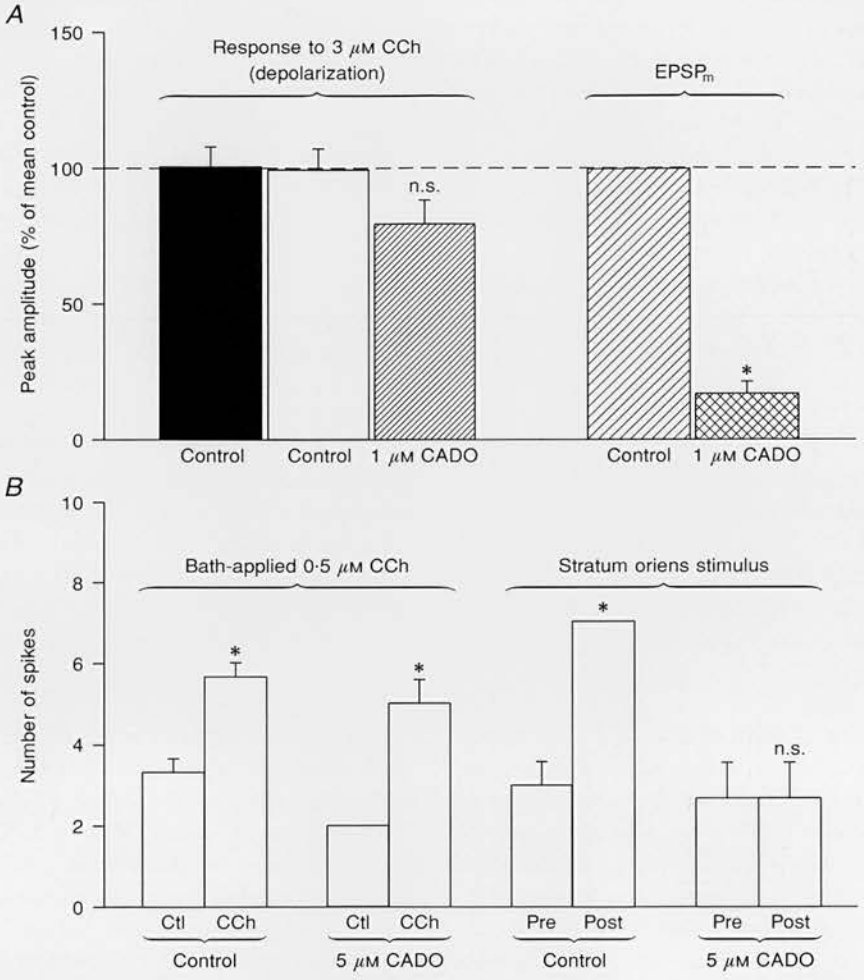

Figure 10. Comparison of the effects of CADO on postsynaptic mAChR-mediated responses evoked by endogenous ACh with those evoked by carbachol

A, pooled data (left) for the peak amplitude of the two carbachol (3 μM)-induced depolarizations prior to the application of CADO (Control) and one depolarization in the presence of 1 μM CADO plotted as a percentage of the mean value for the responses prior to CADO application ($n = 4$; data as generated in Fig. 9*A*); and pooled data (right) for the peak amplitude of the $EPSP_m$ evoked in the presence of 1 μM CADO plotted as a percentage of control ($n = 8$; data generated as in Fig. 3). Note that the same concentration of CADO depressed the $EPSP_m$ to a much greater extent than it did the carbachol-induced depolarization. The small reduction in the size of the carbachol-induced depolarization in the presence of CADO is not statistically significant and can be explained largely on the basis of the decrease in input resistance induced by CADO in this group of cells. In contrast, CADO significantly depressed the $EPSP_m$. *B*, pooled data (left) for the number of action potentials fired during a depolarizing step in the absence (Ctl) and presence (CCh) of 0·5 μM carbachol plotted for control medium and for medium containing 5 μM CADO ($n = 3$; data generated as in Fig. 9*B*); and pooled data (right) for the number of action potentials fired during a depolarizing step 1 s before (Pre) and 2 s after (Post) a subthreshold stimulus in the absence and presence of 5 μM CADO ($n = 3$; data generated as in Fig. 6). Statistical significance was tested for CCh *versus* Ctl and Post *versus* Pre data for each of the paired data sets. Note that CADO completely inhibited the increase in number of action potentials fired following subthreshold afferent stimulation in stratum oriens without affecting the increase evoked by carbachol application. Means ± S.E.M.; * $P < 0·05$; n.s., not significant.

superimposed on glutamate- and GABA-mediated synaptic potentials. Here we have shown that a slow EPSP can be evoked by a single stimulus in the presence of ionotropic glutamate and GABA receptor antagonists without the need for acetylcholinesterase inhibitors. This EPSP is mediated by activation of mAChRs since it was blocked by atropine and unaffected by (+)-MCPG. Its waveform varied considerably between neurones and could not be readily correlated with differences in any of the other electrophysiological characteristics of the neurones in which it was recorded. One notable difference, however, between the $EPSP_m$ recorded in this study and those recorded previously was its large magnitude, even when evoked using just a single stimulus. The reason for this is unclear but it is possible that the amino acid receptor antagonists may relieve an inhibitory influence on the activation of this synaptic potential. Alternatively, in some cells, a number of voltage-activated conductances may boost the apparent magnitude of the $EPSP_m$ (Fraser & MacVicar, 1996). However, preliminary observations that the Ca^{2+} channel antagonist nitrendipine (10 μM) has little or no effect on the $EPSP_m$ suggest that a Ca^{2+}-dependent plateau potential does not necessarily contribute to the $EPSP_m$. Whatever the case, the response kinetics of the $EPSP_m$ were, in general, similar to those reported for $EPSP_m$s evoked by high frequency bursts of stimuli (Cole & Nicoll, 1984; Gähwiler & Brown, 1985; Pitler & Alger, 1990) as well as pure spontaneous and evoked metabotropic glutamate receptor-mediated EPSPs (Charpak & Gähwiler, 1991; Bianchi & Wong, 1995). As with these EPSPs, the $EPSP_m$ described here was accompanied by a reduction in spike frequency adaptation. This effect was mimicked by physostigmine, presumably because this drug increased extracellular levels of spontaneously released ACh by inhibiting acetylcholinesterase activity (Cole & Nicoll, 1984; Azouz *et al.* 1994). A feature of the $EPSP_m$, however, that hampered its investigation was that it could be evoked reproducibly only every 5–10 min. As such, relatively long intracellular impalements were required to obtain meaningful data. This contrasts with metabotropic glutamate receptor-mediated EPSPs which can be evoked reproducibly every 30–60 s (Gerber, Lüthi & Gähwiler, 1993). The reason(s) for the protracted fatigue of the $EPSP_m$ is unclear but by comparison with previous studies it appears to be independent of the stimulation protocol used to evoke it, i.e. a high frequency train *versus* a single stimulus (Cole & Nicoll, 1984; Pitler & Alger, 1990).

Adenosine receptor-mediated depression of postsynaptic responses to synaptically released ACh

To date, few studies have addressed the mechanisms by which mAChR-mediated synaptic transmission is regulated. In one study, the neuropeptide galanin was reported to depress the $EPSP_m$ in the ventral hippocampus by 60% via a presynaptic mechanism (Dutar, Lamour & Nicoll, 1989). Here we have provided compelling evidence that adenosine also inhibits the $EPSP_m$ and that, in addition, it prevents the reduction in spike frequency adaptation mediated by synaptic activation of mAChRs. No distinction was made between dorsal and ventral hippocampus in the present study and, as such, these depressant actions of adenosine are likely to operate throughout the entire CA1 region of the hippocampus. Pharmacological analysis of these effects revealed that they were mediated by adenosine A_1 receptors and that activation of these receptors was capable of abolishing both the $EPSP_m$ and synaptically induced mAChR-mediated reduction in spike frequency adaptation. This is analogous to the situation at glutamate synapses where adenosine A_1 receptors also completely inhibit both AMPA and NMDA receptor-mediated synaptic transmission (Thompson *et al.* 1992; De Mendonça & Ribeiro, 1993). The IC_{50} value for the CADO-induced depression of the $EPSP_m$ is slightly less than that observed for inhibition of AMPA receptor-mediated EPSPs but is similar to that for NMDA receptor-mediated EPSPs (De Mendonça & Ribeiro, 1993; Dunwiddie & Diao, 1994). This possibly reflects the greater need for control of cholinergic and NMDA receptor-mediated synaptic inputs because of their much greater influence on postsynaptic excitability. Indeed, a small degree of adenosinergic inhibition is active tonically as suggested by the enhancement of the $EPSP_m$ by DPCPX. This tonus is greatly enhanced when the metabolism of adenosine is impaired by 5-IT, as is also reported for glutamate synapses (Pak *et al.* 1994). However, the source of the endogenous adenosine tonus is unclear. One possibility is that it is due, at least in part, to non-specific accumulation of adenosine due to cell damage resulting from slice preparation (Thompson *et al.* 1992). Another possibility is that adenosine is released following afferent stimulation (Mitchell, Lupica & Dunwiddie, 1993), e.g. from GABAergic interneurones (Manzoni, Manabe & Nicoll, 1994). Whatever the case, there is considerable evidence that the level of extracellular adenosine in slices is comparable to that in the CNS *in vivo* (Zetterström, Vernet, Ungerstedt, Tossman, Jonzon & Fredholm, 1982; Fredholm, Dunwiddie, Bergman & Lindström, 1984; Dunwiddie & Diao, 1994). As such, it is likely that in the intact animal cholinergic synaptic inputs are tonically inhibited by circulating levels of adenosine.

Locus of the adenosine A_1 receptor-mediated depression of the $EPSP_m$

Since adenosine A_1 receptors are found in abundance both pre- and postsynaptically in the CA1 region of the hippocampus, it is possible that adenosine inhibits mAChR-mediated postsynaptic responses by activation of one or both populations of these receptors (Greene & Haas, 1985; Gerber *et al.* 1989). That the concentration–response relationship for the CADO-induced depression of the $EPSP_m$ most closely paralleled that for the depression of the AMPA/kainate receptor-mediated EPSP, but not that for the postsynaptic hyperpolarization, indirectly favours a presynaptic locus of the adenosine A_1 receptor-mediated depression. In this respect, at least three additional lines of evidence suggest the involvement of a presynaptic depressant

mechanism: (i) CADO can depress the $EPSP_m$ even at concentrations that do not affect postsynaptic passive membrane properties, (ii) CADO does not significantly inhibit carbachol-induced postsynaptic responses, and (iii) both the $EPSP_m$ and mAChR-mediated postsynaptic reduction of spike frequency adaptation evoked by subthreshold stimulation, which result from the inhibition of distinct K^+ conductances (Madison *et al.* 1987), are inhibited by CADO. In addition, neurochemical data from both hippocampal synaptosomes and slices strongly indicate the existence of a presynaptic adenosine A_1 receptor-mediated inhibition of [^{3}H]ACh release (Cunha, Milusheva, Vizi, Ribeiro & Sebastião, 1994). When taken together these data provide convincing evidence that a significant proportion of the adenosine A_1 receptor-mediated inhibition of the $EPSP_m$ and associated reduction in spike frequency adaptation occurs by presynaptic inhibition of ACh release. Such a mechanism has previously been proposed for cholinergic synapses at the neuromuscular junction where activation of adenosine A_1 receptors inhibits the average number of quanta of ACh released from the nerve terminal without affecting the size of each individual quantum (Ginsborg & Hirst, 1972; Silinsky, 1984).

It is conceivable, however, that adenosine may also act postsynaptically to depress mAChR-mediated postsynaptic responses. In this respect, a direct interaction between the transduction mechanisms activated by adenosine A_1 receptors and mAChRs seems unlikely as adenosine A_1 receptors classically couple to the G-protein G_i which inhibits adenylate cyclase activity and neither the $EPSP_m$ nor the mAChR-mediated reduction in spike frequency adaptation are affected by cAMP mimetics (Madison *et al.* 1987; R. A. Morton, unpublished observations) or antagonists of protein kinase A (Pedarzani & Storm, 1993). However, negative interactions between adenosine A_1 receptors and the M_1 mAChR-mediated inhibition of the M-current have been reported in the superior cervical ganglion (Connolly & Stone, 1995). Despite this, the M-current is unlikely to account for the actions of synaptically activated muscarinic receptors in the hippocampus (Madison *et al.* 1987) even though pyramidal neurones are thought to express both the M_1 and M_3 mAChR subtypes (Dutar & Nicoll, 1988; Pitler & Alger, 1990; Segal & Fisher, 1992). These receptors classically couple through the pertussis-toxin insensitive $G_{q/11}$ family of G-proteins to phospholipase C (PLC), the activity of which can, in some cells, e.g. aorta, be inhibited by adenosine A_1 receptors. However, it is unclear whether activation of PLC fully accounts for mAChR-mediated depolarization and reductions in spike frequency adaptation in the hippocampus (Muller & Misgeld, 1986; Dutar & Nicoll, 1988; Colino & Halliwell, 1993). Whatever the case, the inability of CADO to abolish carbachol-induced responses would suggest that this mechanism or a membrane-delimited interaction between the adenosine A_1 receptor-mediated signal transduction mechanism(s) and the mAChR-coupled K^+ conductances is unlikely to account for the depressant effects of adenosine A_1 receptors on the $EPSP_m$ and reduction in spike frequency adaptation in response to subthreshold stimulation (see Fig. 10*B*). However, adenosine, by activating postsynaptic K^+ conductances, will restrict the magnitude of the $EPSP_m$ recorded at the soma, to some extent, by the shunting of membrane currents as well as hyperpolarization towards the reversal potential of the $EPSP_m$ (Cole & Nicoll, 1984).

Implications for adenosine receptor-mediated inhibition of the $EPSP_m$

The established role of adenosine in regulating glutamate but not GABA-mediated synaptic transmission has implicated this neuromodulator as a neuroprotective agent (Thompson *et al.* 1993). The present study identifies another dimension of adenosine-mediated regulation of synaptic excitability in the hippocampal CA1 region, viz. inhibition of mAChR-mediated synaptic responses. This regulation is likely to be of relatively minor importance under normal physiological conditions as the septohippocampal cholinergic synaptic input is important in learning and memory formation *in vivo* (Decker & McGaugh, 1991; Hasselmo & Bower, 1993). However, during seizures or hypoxic and ischaemic episodes, when extracellular adenosine levels are raised, adenosine A_1 receptors may restrict cholinergic synaptic transmission to levels that are below that which is damaging to neurones.

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